ANNUAL REPORT
OF
PROGRAM ACTIVITIES
NATIONAL INSTITUTES OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES
Fiscal Year 1975

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National Institute of Arthritis, Metabolism,
" and Digestive Disease
Report of program activities

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Fiscal Year 1975

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ANNUAL PROJECT REPORTS

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ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

National Institute of Arthritis, Metabolism, and Digestive Diseases

Fiscal Year 1975

by

Dr. J. E. Rall

NIAMDD remains scientifically in good health as evinced by a long list of publications, honors awarded it members and its attractiveness to young scientists.

Several long term trends are evident as one scans annual reports over the last decade:

1) There is an ever increasing complexity and sophistication in the instrumentation employed. This ranges from mass spectrometers to very high field nuclear magnetic resonance instruments and surely includes the pervasive and very powerful dedicated small computors. The results of these newer instruments are to make more quickly available more detailed analyses and in some instances they permit identification and structural insights into complicated compounds which previously were impossible to acquire. Another less obvious implication of these newer machines is a marked increase in the cost of doing this kind of research because of the high costs of the instruments themselves.

2) Another long term trend is an increasing interest in problems of human physiology and disease by even the most basic of scientists. This is indeed, an awareness of the importance of relevance and practicability which society seems to wish. It is also the result of the accumulation of a large amount of basic chemical and physical data in what appeared to be exotic or esoteric materials so that now the more complicated materials of human or animal origin may be studied.

Other long term trends not yet visible may also be mentioned. First amongst these is the gradual dismemberment of the intramural research program of NIAMDD. A decrease in intramural positions from over 500 in 1968 to 418 in 1975 is not yet evident in this report, but it should be apparent that a 16% reduction in staff will produce some reduction in results. Less apparent is the problem of continuing morale and enthusiasm as an organization is gradually decreased in size, and additionally retaining the most productive and original scientists under these conditions. Not to be ommitted is public, administrative and congressional pressures or mandates to institute or expand some programs while the entire program is systematically reduced. We have been able to effectuate a marked increase in the size of the Branch devoted to gastroenterology and liver disease in the last three years, partly in response to these pressures but largely because it was an important area, so far inadequately represented. Another increase has occurred in the group studying diabetes mellitus. These increases, however, have occurred during a time of personnel reduction. The effect on other programs, therefore, has been even more serious than overall statistics show.

It is unclear whether instructions to do more work in certain fields coupled with a reduction in resources has been carefully considered or is merely a concatenation of multiple and different interests converging at the same time on the same organization. It is clear, however, that it is no longer possible to do more and different research with less resources.

ANNUAL REPORT SUMMARY

LABORATORY OF NUTRITION AND ENDOCRINOLOGY, 1975

Dr. Herbert A. Sober, Chief of the Laboratory since 1968, died this past year. His untimely death was a sorrowful event that was deeply felt both in the Laboratory and in the scientific community within which he was an illustrious member. Dr. Sober will long be remembered for his great humanity, his many contributions to science, and his dedication to the training and scientific advancement of many young scientists.

Dr. Martin Rodbell has been appointed Acting Chief of the Laboratory. The Section on Membrane Regulation moved from its previous quarters in Bldg. 10 to newly built laboratories in Bldg 6.

PILOT PLANT UNIT

Large-scale Processing of Biological Materials

During the past year, despite several shutdowns to the new facilities in Bldg 6 for corrections, changes, and additions, a total of 264 requests were processed for investigators: NIAMDD 132: NICHD 51; NCI 23; BB 22; NHLI 8; NIMH 7; NIAID 4; NINCDS and NIDR 3 each; DRS 2; and others 9. 126.3 Kg quantities (29,900 liters of cultures) of 18 organisms were produced. The microorganisms grown during this period include wild type and mutant strains of *Aerobacter aerogenes, Bacillus amyloliquefaciens, Bacillus pumilus, Bacillus subtilis, Bordetella pertussis, Citrobacter sp., Erwian herbicola, Escherichia coli, Haemophilus aphrophilus, Haemophilus haemolyticus, Hameophilus influenzae, Hameophilus parainfluenzae, HeLa cells, Klebsiella cloaceae, Pneumococcus sp., Pseudomonas putida, Pseudomonas sp., Salmonella typhimurium.* Some of the organisms were used as hosts for the production of bacteriophage fd, MS 2, Ø 80, lambda and T4, others were used to produce extracellular enzymes. Microorganisms were grown 94 times in the two 10-liter, 55 times in the 50-liter; 51 times in the 300-liter and 10 times in the 1100-liter fermentors. Of 210 fermentations 24 (11%) were contaminated with 19 of these traced to the inocula supplied by the requesting investigators.

Studies, formerly conducted with the NICHD, were continued with the Division of Bacterial Products, Bureau of Biologics, for polysaccharide production by *H. influenzae,* isolation of the enterotoxin proteins from special strains of *E. coli,* and preparation of the protective antigen of typhoid fever from a non-pathogenic organism, *Citrobacter.* Spheroplasts were produced for the NHLI from *E. coli,* using 10-, 50-, and 300-liter fermentors in series. The Gaulin laboratory homogenizer was used 35 times to rupture cell suspensions of yeast or bacterial cells.

Large scale processing activities consisted of polynucletoide phosphorylase form 1 kg (dry) *Micrococcus lysodeikticus* cells and ^{32}P labeled *Vitellogenin* from chicken serum for NCI, histamine methylating enzyme from 1.75 kg guinea pig brains for NHLI, isolation of the enzyme Barnase and its inhibitor Barstar from 1100 lite cultures of *B. amyloliquefaciens* and eight two-gallon preparations of hydroxylapatite, a product preferred by some investigators over the commercial substance for its superior resolving power and relatively fast flow rate.

Various quantities of plant and animal tissue such as 3.3 kg bovine pituitary glands were either dried, ground and/or extracted in the large ovens, mills and related equipment and solutions such as 50 l of Killam rat virus and 25 l of *H.influenzae* b were centrifuged. A 4 l Shikimic acid solution was freeze-dried in the Stokes hot pump lyophilizer and other solutions were conc trated including a 300 l culture supernate to 8 liters in the Pfaudler reactor, and two 280 liter solutions in the new hollow fiber Amicon concentrator each to 10 liters. (D. L. Rogerson, Jr.)

GERM FREE RESEARCH UNIT

Studies in Experimental Nutrition.

Germfree, ex-germfree, and conventional animals are used to study the interrelationship between the host animal and its bacterial flora, and to identify the organisms involved.

The production of tumors in rats by cycasin and related compounds is being studied. The role of intestinal bacteria, the effects of metabolic products of cycasin and related compounds, and the incidence and types of tumors produced in germfree and conventional animals are being examined in an attempt to identify the actual chemical(s) responsible for specific types of tumors and to identify those reactions which are influenced by the animal or by its bacterial flora.

Possible interrelationships between vitamin A and the trace mineral, zinc, are being examined. Zinc somehow functions in the synthesis of retinal-binding protein (RBP) which then mobilizes and transports the vitamin as a retinal-RBP complex. Growth, which is zinc-dependent, has a marked effect upon the mobilization and transport of vitamin A. Studies are in progress to determine whether the effects of zinc on vitamin A levels and growth are related to other factors such as diet. Other relationships between vitamin A and zinc appear in Kwashiorkor liver diseases and infections. (J. Smith, VA Hospital, and E. G. McDaniel).

DEVELOPMENTAL BIOCHEMISTRY SECTION

Analysis of the Active and Inactive Portions of the Eukaryotic Genome.

A number of further steps have been made in understanding the structural differences between repressed and transcribable chromatin fractions eluted early and late, respectively, from ECTHAM-cellulose chromatography. The earliest material eluted appears to be about 50% constitutive heterochromatin, a structural element in the chromosome. It contains large amounts of satellite DNA and is resistant to the action of deoxyribonucleases. The content of satellite DNA in the transcribable sequences borders on zero.

We have interpreted the structures of repressed and transcribable chromatin segments in terms of the recently developed concept of chromatin as consisting of beaded subunits with spacer regions of DNA between. Repressed chromatin has series of beads observable up to at least tetramers with average spacer regions of about 45 base pairs. In contrast, transcribable chromatin exists as longer spacer regions of DNA - averaging about 200 base pairs - and only single or rarely double beads on a given fragment. Constitutive heterochromatin does not appear to have a beaded subunit structure since it is not converted to the 11S subunit particles by the action of deoxyribonuclease II.

Hybridization studies are being employed in efforts to determine if gene segments which we isolate and characterize as transcribable *in vitro* are also *in vivo*. Using rabbit liver chromatin and globin cDNA hybridization saturation levels were observed which are consistent with the elution of three species sequentially from the ECTHAM-cellulose column; constitutive heterochromatin, repressed euchromatin, and transcribed euchromatin. As yet incomplete studies with cDNA made to the polyadenylate containing fraction of rabbit liver polysomal RNA are necessary to establish the identity of this latter fraction. (R. T. Simpson, L. S. Propst).

Protein-nucleic Acid Interactions: Chromatin Structure and Function.

The effects of substitution of 5-deoxybromouridine for thymidine in the DNA of HeLa cells on the metabolism of nuclear RNA has been investigated. While the rate of RNA synthesis measured over long (2 hr) periods is the same for the control and dBrU containing cells; there is a defect at shorter periods. Indeed, at times less than 5 min label period, the dBrU cells synthesize RNA at a rate half that of controls. In contrast to these differences in rates of RNA synthesis, there is no detectable difference between controls and dBrU cells with regard to the size distribution of rapidly labeled, nuclear RNA, the size distribution of that fraction of the hnRNA which contains poly A, or the size of the 3' poly A tails and the internal oligo A spacer regions of the hnRNA. (J. P. Whitlock, R. T. Simpson).

Interactions of Histones with DNA.

Chromatin is acetylated as a native complex with radioactive acetic anhydride, the histones dissociated and totally acetylated with cold acetic anhydride and then digested with trypsin. Peptides are fractionated by two-dimensional fingerprinting, yielding about 65 spots detectable with fluorescamine, about 5-10 more than predicted. Radioactivity is confined to some 9-11 peptides which are among the largest present, judged both by their behavior on fingerprinting and by their elution position on gel filtration. A combination of G-25 Sephadex chromatography and fingerprinting should allow isolation of the peptides of interest in sufficient amount for identification and placing in the known primary sequences of the histones. (L. Tack and R. T. Simpson).

Isolation of Preparative Amounts of Large Oligomers from Viral Nucleic Acids.

S_1 nuclease from *Aspergillus oryzae* was purified to enzymatic homogeneity, importantly removing significant amounts of RNases T_1 and T_2 present in usual preparations of this single-strand specific nuclease. Using this enzyme, it was possible to selectively cleave two tRNA molecules at about their midpoints, producing fragments of 12,000 and 14,000 daltons. The presumption is that cleavage of this molecule, which possesses extensive base-paired double-stranded structure, occurs at the codon loop, known from X-ray studies to be in a more exposed area and single stranded in conformation, although this remains to be proven by sequence studies of the cleavage products.

An important technical development has been made in the generation of methodology which allows the nondestructive, rapid, quantitative determination of the distribution of ^{32}P radioactivity in gel electrophorograms. Utilization of a gas flow strip chart counter with minor modifications in the counting geometry allows facile determination of radioactivity distribution in polyacrylamide gels. This method should be of significant assistance in nucleic acid sequencing studies. (G. W. Rushizky, J. Mozejko).

Structure and Function of Barnase and Barstar, an Extracellular Ribonuclease and its Intracellular Inhibitor from *Bacillus amyloliquefaciens*.

Sodium dodecyl sulfate (SDS) induces a reversible, two-state conformational transition in barnase. Below the transition, no SDS is bound and above the transition 14 molecules are bound per molecule of enzyme. This number is constant at less than 1 mM concentrations of SDS and is thought to be significant in the mechanism of interaction of this detergent with proteins.

Extensive modification of a JEOLCO amino acid analyzer to use o-phthalaldehyde as a detection reagent has allowed marked improvement in the sensitivity of amino acid analysis. Compared to fluorescamine, o-phthalaldehyde has the advantage of being stable in aqueous solution, hence only requiring a two stream mix as compared to three streams for fluorescamine, and further, is more sensitive. Microbore columns and pressure controlled flows allow extreme sensitivity (analyses routine with 50 picomoles of amino acid residue) and stability. (R. W. Hartley).

Metabolic Studies on Chromatin: Synthesis, Assembly and Turnover of Chromatin Constituents.

Non histone chromosomal proteins have been considered to be the prime candidates for regulators of genetic activity in eukaryotic cells and hence have been thought to be metabolically active, with significant turnover, although this question has never been experimentally approached previous to this time. HeLa cells were labeled for either a full generation or 30 min, both in random growth and during G1 and S phase in synchronized populations. Turnover was investigated during several generations growth in the absence of radioactive amino acid. In no case was any turnover detected (estimated limit of detection 5-10% per generation). Thus, nonhistone proteins are metabolically as stable as histones, suggesting that many of these proteins may serve structural roles in the chromosomes.

Studies concerning chromatin assembly mechanims have been carried out using both cells treated with cycloheximide (CH) to block protein synthesis while allowing continued DNA synthesis at 20% of control rates and untreated cells wherein nascent chromatin DNA is more sensitive to micrococcal nuclease or DNase I, having a resistance to digestion exactly intermediate between mature chromatin and protein-free DNA. In control cells, this difference is lost over about 15 min, while in CH cells, three hours after drug removal are required for nuclease resistance to rise to control levels. In both cases, this is the time frame required for processing of intermediate sized DNA fragments to complete replicon sized pieces. The data suggest that histones are not added onto newly synthesized DNA until the time of completion of synthesis of a full replicon. Those histones that remain associated with newly synthesized DNA interact in a similar fashion to those in mature chromatin, as judged by the size distribution of oligonucleotides present in a limit digest with micrococcal nuclease.

In contrast to the expectation from these data that newly synthesized DNA would be protein deficient and hence band in CsCl at a greater density than fixed native chromatin, both CH chromatin and nascent chromatin band at densities lighter than control samples. The basis for this behavior is not known. The duration of this state for control samples is only about one minute, but the persistence of the light state for CH chromatin implies that further protein synthesis is required for the transition from this state to mature chromatin. (R. L. Seale).

Biochemistry of Pituitary Glycoprotein Hormones.

TSH, LH, and FSH are absorbed from extracts of lyophilized pituitary onto Concanavalin A - Sepharose in high yield. The hormones are eluted with α -methyl-D-glucoside prior to their fractionation by other conventional methods. Bovine TSH prepared by this affinity chromatographic method compared favorably to preparations made by conventional methods in biological activity, and was recovered in higher yield. Disaggregation of whale and bovine TSH and LH was studied by ANS fluorescence. Conditions for complete disaggregation of bovine TSH were not defined as yet. Preliminary evidence suggests that hybrid, active hormones may be formed from whale and bovine subunits in combination. (P. G. Condliffe).

SECTION ON MEMBRANE REGULATION

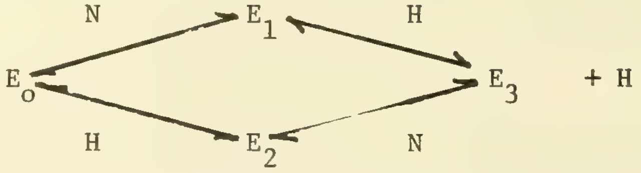
Effects of Gpp(NH)p on Adenylate Cyclase in Eukaryotic Cells.

Previous studies have shown that both GTP and glucagon are necessary to activate adenylate cyclase in hepatic plasma membranes. Gpp(NH)p, a non-metabolizable synthetic analog of GTP, activates adenylate cyclase in membranes isolated from a variety of eukaryotic cells. The effects of Gpp(NH)p are exerted through the same site as GTP, occur independently of the nature of the hormone receptors coupled to these systems or in the absence of hormones, and are seen with solubilized preparations of the enzyme. Activation by Gpp(NH)p involves a time-dependent process which is diminished or abolished in the presence of hormones. In some cases Gpp(NH)p and hormones synergistically act to give enzyme activities hitherto unrealized with GTP and hormones. Gpp(NH)p has no effect on bacterial systems thus far examined.

Gpp(NH)p is a powerful tool for investigating the role of the nucleotide regulatory site in the activation process and for assessing the effects of hormones on this process. (M. Rodbell, M. C. Lin, Y. Salomon, J. P. Harwood, and C. Londos).

Actions of Catecholamines and Gpp(NH)p on Frog Erythrocyte Adenylate Cyclase.

Catecholamines stimulate adenylate cyclase via a beta-receptor; propranolol, a powerful beta-blocking agent, specifically blocks the actions of catecholmines on the erythrocyte system. Gpp(NH)p also stimulates enzyme activity. Catecholamines and Gpp(NH)p synergistically cause marked increases in enzyme activity. Pretreatment of the enzyme with hormone and Gpp(NH)p, followed by washing the membranes prior to enzyme assay, results in a high rate of enzyme activity. Similar phenomena have been noted also with the adrenal and hepatic cyclase systems in the rat. By various manipulations of the time sequence of addition of catecholamines, propranolol, and Gpp(NH)p it was determined that the enzyme, once activated by Gpp(NH)p and catecholamine, remains in a persistent activated state that is no longer inhibited by propranolol or stimulated further by the addition of Gpp(NH)p or catecholamines. In fact, addition of catecholamines to the activated enzyme (previously washed free of bound catecholamines) resulted in the same dose-dependent manner seen for the stimulatory effects of the catecholamines. The following model is suggested by these observations



in which N and H represent Gpp(NH)p and catecholamine, respectively, and E represents various enzyme states having different activities. In this model, it is suggested that both N and H produce a high activity state of the enzyme (E_3) from which the hormone dissociates, leaving a state that is fully active even in the absence of hormone. The inhibitory effects of hormone may be due to establishment of a new equilibrium between the various states of the enzyme system. This model is also consistent with the actions of glucagon and GTP on the hepatic adenylate cyclase system in the rat. (M. Schramm and M. Rodbell).

Structure-Activity Relationships for Guanine Nucleotides.

Binding and action of guanine nucleotides on the hepatic adenylate cyclase system in rat plasma membranes has been investigated. Action requires three phosphate groups at the 5'-position of ribose (or 2' and 3' deoxyribose); phosphate groups can be bridged by a methylene or imido groups, and a thiol group can be attached to the terminal phosphate to give potent stimulatory effects. Substitutions on the ribose group or of sugars at the terminal phosphate results in loss of both binding and action. GDP binds to the nucleotide regulatory site with approximately the same affinity as GTP or Gpp(NH)p. However, GDP does not activate adenylate cyclase and, indeed, inhibits the actions of glucagon and competitively inhibits the actions of Gpp(NH)p or GTP on the system. These findings provide strong evidence for the importance of the nucleotide regulatory site in the activation of the enzyme and its response to hormones. (Y. Salomon and M. Rodbell).

The Fate of GTP and Gpp(NH)p at the Nucleotide Regulatory Site.

Activation of adenylate cyclase systems by GTP, in contrast to Gpp(NH)p, requires the presence of hormones. By examining the fate of GTP and Gpp(NH)p in the incubation medium and when bound to hepatic and fat cell plasma membranes, evidence was provided that GTP is hydrolyzed at the nucleotide regulatory site to GDP. By contrast, Gpp(NH)p is not metabolized at this site. Such findings suggest, therefore, that differences in the ability of GTP and Gpp(NH)p to activate the enzyme in the absence of hormones relate to the susceptibility of the terminal phosphate of GTP to hydrolysis. The possibility is raised that glucagon and other hormones exert their effects by increasing the affinity of the regulatory site for GTP, by altering the rate of degradation of GTP and dissociation of degradation products from the regulatory site, or by a combination of these processes. Since GTP is thought to be the natural regulatory nucleotide for cyclase in the intact cell, hydrolysis of GTP at the regulatory site would require continuous presence of adequate concentrations of GTP for activation of adenylate cyclase; GDP, which also binds to the site and inhibits enzyme activity, may also serve a regulatory capacity on the enzyme system. (Y. Salomon and M. Rodbell).

Computer Analysis and Model Fitting of Steady State Kinetics of Adenylate Cyclase.

In collaboration with Drs. Mark Rendell and Mones Berman (Laboratory of Theoretical Biology, National Cancer Institute), modeling by the SAAM program of the steady state kinetics of the hepatic and adrenal cyclase systems has been carried out. Of several models considered and tested, the only model giving excellent fits with experimental data was one in which the enzyme is accessible to three fundamental states of activity, designated as E, E', and E". The catalytic site of these states interacts with ATPMg (the productive form of substrate), ATPH (an inhibitory form of substrate which is referred to as protonated substrate), and Mg^{2+}, which to the extent that it binds to the catalytic site and competes with the ATPMg complex, is also an inhibitor. These kinetic parameters along with V_{max} were evaluated for each of the transition states using an iterative, computer-fitting procedure. The principle conclusions drawn from these studies are as follows: 1) Guanine nucleotides convert E, a low activity, ground state of the enzyme to E'; 2) E' has the property in both the rat and adrenal cyclase systems of having a very high affinity for protonated substrate relative to E and E". In addition, the adrenal system exhibits an increased affinity for both Mg^{2+} and ATPMg without significant changes in V_{max} relative to state E; 3) E', when liganded with Gpp(NH)p, spontaneously isomerizes to E", which has the property of a higher V_{max} than E and E' and a considerably lower affinity for protonated substrate, the result being an increased expression of catalytic activity by this state; 4) glucagon (the hepatic system) and ACTH (the adrenal system) accelerate the rate of transition of E' and E". Thus, the hormones convert the enzyme from a state which exhibits tight binding of protonated substrate and Mg at the catalytic site to a state having increased V_{max} and marked decreases in affinity for these ligands.

The transition state model for the actions of guanine nucleotides and hormones provides an explanation for the effects of magnesium ion and pH on enzyme activity, the transient kinetics of Gpp(NH)p-activation, the effects of hormones on the transient kinetics of nucleotide activation, and the interdependent and synergistic effects of GTP or Gpp(NH)p in combination with hormones. The model has also given insights for future studies of the structure-function of the various components of the cyclase system. For the first time, a membrane-bound complex regulatory enzyme system has been systematically evaluated to give a satisfactory resolution of the possible conformational states of the enzyme in the absence and presence of regulatory ligands.

An important finding was the marked difference in the properties of E' between the adrenal and hepatic cyclase systems. Both systems displayed essentially identical kinetic parameters for states E and E" whereas E' differed markedly in the affinities for magnesium ion. These differences suggest that, though the process of catalysis is identical and the actions of guanine nucleotide and hormones are quite similar on these systems, the intermediate transition states may have very different structural properties. It remains for future studies to determine the nature of these putative structural differences in E'. (M. Rodbell, Y. Salomon, M. C. Lin, and C. Londos).

Effects of Guanine Nucleotides on Fat Cell Adenylate Cyclase.

It was shown previously that GTP stimulates adenylate cyclase activity in the presence of the various hormones (glucagon, secretin, ACTH, and catecholamines) operating on this multi-receptor system, but paradoxically inhibits activity in the absence of hormones. Gpp(NH)p also inhibits enzyme activity by a transient process that is followed by a marked stimulation of enzyme activity. Hormones abolish the transient inhibitory phase of Gpp(NH)p action and accelerate the rate of transition of the enzyme to its fully active state. Transient inhibition by Gpp(NH)p is abolished by increasing the concentration of magnesium ion and pH, conditions which reduce the concentration of protonated substrate. Protonated substrate, as found with the liver and adrenal cyclase systems, is a potent inhibitor of the fat cell system. The inhibitory effect of GTP on basal activity is also abolished by reducing the concentration of protonated substrate. Thus, as predicted by the three state transition model described for the adrenal and hepatic cyclase systems, GTP and Gpp(NH)p induce the formation of a transition state (E') that is highly susceptible to inhibition by protonated substrate. The complex and seemingly paradoxical behavior of the multi-receptor fat cell system has provided strong supportive evidenc for the three state model. It seems that the intermediate transition state E' in the fat cell differs from the corresponding state in the adrenal and hepatic cyclase systems, as evidenced by the marked inhibition of enzyme activity induced by the guanine nucleotides on the fat cell system. (M. Rodbell).

Structure-function Relationships in Glucagon and Secretin.

Knowledge of the structural or topological features of a hormone required for recognition and action is of fundamental importance for understanding the basis of hormone action. The affinity and activity of glucagon for the hepatic adenylate cyclase system has been compared with two chemically modified derivatives of the hormone. Des-(Asn^{-8}, Thr^{29})-homoserine-lactone^{27}-glucagon was prepared by CNBr treatment and extensively purified; the derivative lost activity and affinity equally by about 50-fold relative to native glucagon. By contrast, des-His^{1}-glucagon, prepared by an insoluble Edman degradation procedure and freed of native glucagon, displayed a 15-fold decrease in affinity but a 50-fold decrease in activity relative to native hormone. Therefore, the role of the residues at the COOH-terminal region of glucagon is strictly for recognition by the glucagon receptor, while the NH_2-terminal histine residue plays an important role both in recognition and particularly in the expression of hormone action.

Chemical synthesis of secretin, a hormone that has a similar primary sequence of residues to that of glucagon in the NH_2-terminal region, has been completed by Dr. Victor Hruby. This study will continue as a collaborative project with the goal of replacing suitable residues of the hormone for studies of structure-function relationships in this hormone. A plentiful supply of secretin will also allow X-ray crystallographic studies of the molecule in collaboration with Dr. T. Blundell at the University of Sussex in England. (M. C. Lin).

Binding of Glucagon at its Receptor.

Three aspects of the interaction of glucagon ith rat liver plasma membranes have been described previously: 1) Binding of ^{125}I-glucagon; 2) activation of a glucagon-specific adenylate cyclase system; and 3) inactivation of glucagon by a "glucagon-specific inactivating mechanism". The "inactivating process" has been found to be an artifact which can be attributed to damage of ^{125}I-glucagon by beta radiation and differential binding of the damaged labeled glucagon and native glucagon to receptor sites in the membrane. A highly sensitive method, using the specific activation of adenylate cyclase by glucagon, was developed for measuring the uptake of glucagon by receptors without the use of labeled hormone. With this procedure it was found that the amount of glucagon disappearing from the medium in the presence of liver membranes correlates well with the amount of glucagon taken up by the membranes. The rate of equilibration of native hormone with the receptor sites is considerably greater than the rate observed with labeled hormone; the amount of receptor sites calculates to be 3 to 5 times greater than estimated from the labeled hormone binding procedure. As was previously observed in studies with labeled glucagon, guanine nucleotides caused marked decreases in the amount of glucagon taken up by the receptors.

These studies have revealed the problems one may encounter with the use of ^{125}I-glucagon and possibly similarly labeled hormones when studying the kinetics of hormone binding. They have also provided a standard for determining the efficacy of other types of labeled hormone preparations. In view of the recently discovered role of glucagon in the hyperglycemia of the diabetes syndrome, and with the expectation that studies on the actions of glucagon will be intensified, the importance of such information is obvious (C. Londos).

Actions of Hydroxybenzylpindolol (HBP) on Fat Cell Adenylate Cyclase.

Pindolol is a potent beta-blocker of the catecholamine-sensitive cyclase system in turkey erythrocytes. HBP also competitively inhibits the actions of catecholamines on this system with an affinity three orders of magnitude higher than the catecholamines. Studies of the actions of pindolol and HBP on fat cell cyclase gave rise to the paradoxical finding that HBP is an activator of this system with a 25-fold higher affinity than catecholamines. Pindolol is a weak agonist on this system. Propranolol, a classical beta-blocker, only acts as an antagonist. Hydroxybenzylpropranol exerts weak stimulatory effects on the enzyme system but appears to bind to the catecholamine receptor with the same affinity as hydroxybenzylpindolol. These findings suggest that pindolol, propranolol, and the corresponding hydroxybenzyl derivatives bind to the same catecholamine receptor. The finding that HBP is an activator has broad implications for the structure-function relationships required for binding and action at the beta-receptor of the fat cell system. The basis of the different effects of HBP on the turkey erythrocyte and rat fat cell beta-receptors remain unknown but it seems likely that they may reflect either differences in the structure of the receptors or their structural relationship to adenylate cyclase. (H. Yamamura and M. Rodbell).

NUTRITIONAL BIOCHEMISTRY SECTION

Biochemical Studies Related to Nutrition.

Studies of factors regulating the distribution of vitamin E in the body have shown that the amount of the vitamin deposited in tissues is inversely related to the concentration of plasma total lipids. Animal models were rats made hypolipemic by feeding orotic acid and genetically obese rats which develop spontaneous hyperlipemia. Another factor is the degree of body adiposity. At the same vitamin E intake, obese animals have higher total stores of the vitamin with most of it in the adipose tissue. As a result, organ tissues have lower concentrations than in non-obese animals. These studies emphasize the complexity of evaluating body vitamin E status.

To obtain evidence of the current vitamin E status of individuals, 5 tissues from 6 human autopsies were analyzed for α- and γ-tocopherols and fatty acids. The molar ratio of tocopherol:polyunsaturated fatty acids, an index of vitamin E adequacy, was found to be more favorable than ratios previously found in tissues from rats ascertained to have a satisfactory vitamin E status. (J. Bieri and R. Evarts).

Biochemical Studies of Cytochromes and Sub-cellular Particles.

Glucagon injections into rats were found to produce significant decreases in the amount of mitochondrial cytochromes aa_3, b, c_1, and c and also of cytochrome oxidase. The magnitude of the decreases generally paralleled the decrease in mitochondrial protein. (J. N. Williams).

Biochemical Studies of Hepatic and Intestinal Function.

A technique was developed to detect and quantitate the metabolism of dietary constituents during their transport from the intestinal lumen into the blood and to determine the relative rates of intestinal metabolism of substances from the lumen and from the arterial blood. Results indicate that all ingested glutamate and most glutamine, which together comprise 15-30% of the amino acid residues of dietary orotein, are metabolized to CO_2, organic acids, and other amino acids during intestinal transport. Dietary glutamine and glutamate and circulating glutamine emerge as important respiratory substrates for the small intestine. (H. G. Windmueller).

VITAMIN METABOLISM SECTION

Studies on Folic Acid.

Dihydrofolate reductase catalyzes the reduction of folic acid or dihydrofolic acid to tetrahydrofolate. The enzyme is the site of action of the cancer chemotherapeutic drug, Methotrexate. Beef liver dihydrofolate reductase has been purified to homogeneity using a methotrexate affinity column followed by gel filtration to remove several higher molecular weight proteins and hydroxylapatite chromatography to remove bound substrates. The overall purification is 13,000 fold; the specific activity is 26 units -mg^{-1}, approximately 25 times higher than previously reported. This corresponds to a turnover number of 578 moles of dihydrofolate reduced per mole of enzyme. The enzyme has been shown to be homogeneous by the following criteria: disc gel electrophoresis, SDS gel electrophoresis, velocity sedimentation, equilibrium sedimentation and methotrexate titration. The amino acid composition has been determined. Notable features include a single cysteine, 3 tryptophan, and 3 histidine residues. The molecular weight determined by equilibrium sedimentation is 22,500. The $S^{o}_{20,w}$ is 2.08 x 10^{-13} sec^{-1} and the $D^{o}_{20,w}$ is essentially spehrical. An isoelectric point of 6.08 was measured. (Bernard T. Kaufman).

SECTION ON ENDOCRINOLOGY

Mechanism of Hormonal Regulation of Lipoprotein Lipase Activity in Mammary Gland.

Dietary fatty acids are used by lactating mammary gland for the synthesis of milk triglyceride. They are transported in blood as triglyceride in chylomicrons and their uptake by mammary gland is regulated by lipoprotein lipase. Lipoprotein lipase activity is markedly increased in mammary gland and decreased in adipose tissue during lactation, thus diverting dietary fatty acids from body fat stores to the mammary gland for the synthesis of milk triglyceride. Studies reported earlier showed in lactating rats that these changes in lipoprotein lipase activity are mediated through prolactin secretion by the anterior pituitary gland. Studies by others, in pseudo-pregnant rabbits, showed that the effect of prolactin on lipoprotein lipase in mammary gland requires the continued production of a short-lived messenger RNA.

The present study was initiated to determine the mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland using a mammary tissue preparation, such as explants, capable of responding *in vitro* to prolactin. During the past 6 months, studies have been made in both intact and hypophysectomized immature rats to determine the pattern of hormonal priming (with growth hormone, estrogen, progesterone and glucocorticoids) necessary for a rapid effect of prolactin on lipoprotein lipase in the gland. The findings to date indicate that growth and development of the mammary gland occur in immature animals without any change in lipoprotein lipase activity in the tissue. The possibility that prolactin and other hormones may also be necessary for priming the gland is being investigated. (P. M. Spooner and R. O. Scow).

Effect of Prolactin on Lipoprotein Lipase Activity in Crop Sac and Adipose Tissue of Pigeons.

Crop "milk" is an oily, cheese-like substance, consisting of desquamated epithelial cells, produced in the crop sac of pigeons and doves for feeding their young. Since crop "milk" has a high fat content, 12%, and its formation is dependent on prolactin, the possible role of lipoprotein lipase in this process was investigated in adult pigeons. It was found that prolactin injection ions for 4 days increased lipoprotein lipase activity 9-fold in the crop sac and less than 1-fold in adipose tissue. Prolactin injections also stimulated growth of the crop sac and production of crop "milk". The finding of markedly increased lipoprotein lipase activity in crop sac of prolactin-treated pigeons suggested that blood triglyceride may be used by crop sac for the formation of crop "milk" lipid. (R. O. Scow).

Effect of Prolactin on Blood Lipids in Pigeons.

Since the diet of pigeons is usually low in fat and high in carbohydrate, it is likely that some of the fatty acids in blood triglyceride are derived from synthesis *de novo*. Studies elsewhere showed that fatty acid synthesis in normal and prolactin-treated pigeons occurs primarily in the liver and that fatty acids stored in adipose tissue are derived mostly from triglyceride in blood. It was also found that fatty acid synthesis in liver, the concentration of fatty acids in blood, and accumulation of fat in adipose tissue are markedly increased by prolactin. The proportion of fatty acids in blood derived from diet and that from synthesis in liver, as well as the class of plasma lipoprotein involved in their transport, were not studied. The present investigation was initiated to study these aspects of lipid transport, as well as the source of fatty acids secreted in crop "milk" lipid and the role of lipoprotein lipase in their uptake by crop sac.

It was found that daily injections of prolactin increased the serum triglyceride concentration of pigeons 2-fold in 2 days and 3-fold in 7 days, and doubled the phospholipid and cholesterol concentrations within 7 days. Ultracentrifugation studies showed that most of the increase in triglyceride content of serum occurred in the $d<1.006$ fractions. The startling finding concerning this fraction was that it contained nearly equimolar concentrations of triglyceride, phospholipid and cholesterol, suggesting that it may be composed mostly of remnants of very low density lipoproteins (or chylomicrons). Electron microscopic studies showed that the particles in this fraction ranged in diameter from 125 to 1800 Å, and that some resembled in appearance remnants of chylomicrons produced, *in vivo* and *in vitro*, in this laboratory. (A. A. Ucci, S. S. Chernick and R. O. Scow).

Secretion of Milk Lipid by Mammary Gland.

Morphological observations in mammary gland of lactating rats indicate that the Golgi apparatus plays a major role in the release of milk lipid from mammary alveolar cells. Lipid droplets in the cells become surrounded by Golgi vesicles at the apex of the cells. The membranes of the vesicles then fuse with each other and with the apical plasma membrane of the cells resulting in the secretion of lipid droplets. The Golgi vesicles are also the origin of membranes surrounding milk lipid droplets in the alveolar lumen as well as some of the apical plasma membrane of the alveolar cells. (E. J. Blanchette-Mackie).

Effects of Lipoprotein Lipase on Chylymicrons *in Vitro*.

Earlier studies in this laboratory showed that purified lipoprotein lipase, obtained from bovine milk, readily hydrolyzed triglyceride of chylomicrons to FFA and glycerol if sufficient albumin was present in the medium to bind all of the FFA produced. However, when albumin was limited, it was found that lipolysis was markedly reduced and that FFA and monoglyceride, products of lipolysis, accumulated in the chylomicrons. Structural analysis with transmission electron microscopy (of whole mounts, sections and freeze fracture replices) and scanning electron microscopy showed that these chylomicrons also contained aqueous spaces lined with a lipid monolayer. Sometimes, the aqueous spaces contained whorls of bilayered lamellae which appeared to be continuous with both the surface film of the chylomicron and the lipid monolayer lining the aqueous space. These findings suggest that the monolayer lining the spaces may be an extension of the surface film, although different in composition, formed by the accumulation of FFA and monoglyceride at the triglyceride-water interface, and that this monolayer can extend itself by forming a spiralling fold within the aqueous space. Thus, hydrolysis of triglyceride forms amphiphilic acyl lipids, FFA and monoglyceride, which can locate and move within the interface between lipid and water. It is proposed that this translocation of lipid probably is the principal effect of the action of lipoprotein lipase on chylomicrons and that it is essential for the transfer of fatty acids from chylomicrons across capillary endothelium. (E. J. Blanchette-Mackie and R. O. Scow).

Fatty Liver in Diabetic Rats.

Fatty liver and hypertriglyceridemia, as well as severe diabetes, develop within 24 hours following "total" pancreatectomy in fasting rats. Accumulation of triglyceride in the liver results from increased mobilization of fatty acids from adipose tissue, and the high triglyceride concentration in blood, up to 6 mM, reflects increased lipoprotein secretion by the liver. Electron microscopic study of livers from these animals showed that the lumina of endoplasmic reticulum in the parenchymal cells contained small lipid particles (400 Å) which had structural continuity with membranes of the endoplasmic reticulum. Also, the large lipid droplets within the cells contained, in the triglyceride core, whorled bilayered lamellae which were similar to those found in the aqueous spaces of partially lipolyzed chylomicrons (see above). The lamellae in the lipid droplets also appeared to be continuous with the membranes of the smooth endoplasmic reticulum, whereas the triglyceride formed accumulate in the hydrophobic zones of the membranes, between the lamellae. (E. J. Blanchette-Mackie and R. O. Scow).

Pharyngeal Lipase.

Last year it was reported that a lipolytic activity was found in esophageal and gastric aspirates from normal subjects which was capable of hydrolyzing triglyceride to partial glycerides and FFA at pH 5.5. The lipolytic activity was not present in secretions obtained from the major salivary glands, thus excluding those tissues as a source of the activity found in the esophageal aspirates. It was proposed that in man glands in or near the pharynx secrete a lipase that acts in the stomach to hydrolyze long chain triglycerides to partial glycerides and FFA, and that this was the first step in the digestion of dietary fat. Subsequent studies have shown that the lipolytic products formed, especially monoglyceride and FFA, facilitate the emulsification of triglyceride in the stomach. Recent collaborative studies at the Department of Pediatrics, University of Oulu, Finland indicate that pharyngeal lipase is also present in the neonatal and premature infants. Studies are now in progress to determine the role and site of origin of the enzyme in man. (R. O. Scow)

COLLABORATIVE RESEARCH

Effects of Hormone Metabolism of Adipose Tissue Perfused *in vivo*: with Dr. Vance Kovacev, Department of Physiology, Medical Faculty, University of Skopje, Skopje, Macedonia, Yugoslavia (S.S. Chernick)

Pharyngeal Lipase and its Role in Fat Digestion in Premature and Neonatal Infants: with Drs. Hans Åkerblom and Kauko Kouvalainen, Department of Pediatrics, University of Oulu, Oulu, Finland (R. O. Scow).

Synthesis of Secretin, Glucagon, and Selected Analogs: with Dr. Victor K. Hruby, Department of Chemistry, The University of Arizona, Tucson, Arizona (M. Rodbell).

Metabolic Studies of Vitamin A: with Dr. J. Ganguly, Department of Biochemistry, Indian Institute of Science, Bangalore, India (J. Bieri).

Project No. Z01 AM-15000-05 LNE
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanism of action of hormones and guanine nucleotides on adenylate cyclase systems.

Previous Serial Number: NIAMDD-LNE-7

Principal Investigator: Dr. Martin Rodbell

Other Investigators: Dr. Michael C. Lin, LNE; Dr. Yoram Salomon, LNE; Dr. Constantine Londos, LNE; Dr. Michael Schramm, LNE; Dr. Hirohei Yamamura, LNE, NIAMDD.

Cooperating Units: Dr. Marc Rendell and Dr. Mones Berman, Laboratory of Theoretical Biology, NCI

Man Years:

Total:	6.35
Professional:	6.35
Other:	0

Project Description:

Objectives: To determine the mechanism of action of hormones on adenylate cyclase systems in membranes from various animal cells.

Methods Employed: Cell membranes (primarily plasma membranes) from fat cells, liver cells, and frog erythrocytes are purified by differential centrifugation in sucrose gradients. A new highly sensitive method for assaying adenylate cyclase activity in these membranes has been developed which enables rapid processing of assay medium and determination of extremely low quantities of cyclic AMP formed during kinetic analysis of the enzyme. Various types of chromatographic procedures and methods for measuring metabolism and binding of suitably labeled nucleotides are also employed. Mathematical modeling of the kinetic parameters of the enzyme systems was carried out using the SAAM program developed by Dr. Mones Berman of the Theoretical Biology Laboratory in the National Cancer Institute. An iterative fitting routine was used with standard procedures of analysis of standard deviations.

Major Findings: I. Effects of Gpp(NH)p on adenylate cyclase in eukaryotic cells. Previous studies established that GTP is a primary activator of the hepatic adenylate cyclase system and that glucagon regulates the reactivity of the system with GTP. Using GPP(NH)p, an isosteric analog of GTP that is not susceptible to hydrolysis by nucleotide phosphohydrolases, we have established that the guanine nucleotide regulatory process is present in a large variety of eukaryotic cells. Gpp(NH)p, in contrast to GTP, activates adenylate cyclase systems in frog erythrocytes, salmon erythrocytes, thyroid cells, adrenal cells, fat cells, platelets, and hepatic cells irrespective of the nature of the hormone receptor coupled to these systems. Gpp(NH)p also activates adenylate cyclase in membranes from mutants of adrenal cells that do not exhibit a functional ACTH receptor, indicating that the nucleotide does not activate the system through the hormone receptors. This was further indicated by the findings that Gpp(NH)p activates adenylate cyclase in detergent solubilized preparations which do not have functionally coupled receptors. However, in the presence of a functionally coupled hormone receptor, combination of hormones and Gpp(NH)p results in synergistic effects on adenylate cyclase activity. Thus, Gpp(NH)p is a powerful tool for investigating the role of the nucleotide regulatory site in the activation process and for assessing the effects of hormones on this process.

II. Actions of catecholamines and Gpp(NH)p on frog erythrocyte adenylate cyclase. Catecholamines stimulate adenylate cyclase via a beta-receptor; propranolol, a powerful beta-blocking agent, specifically blocks the actions of catecholamines on the frog erythrocyte system. Catecholamines and Gpp(NH)p synergistically increase enzyme activity. By various manipulations of the time sequence of addition of catecholamines, propranolol, and Gpp(NH)p during pretreatment, it was determined that the enzyme, once activated by Gpp(NH)p and catecholamine, remains in a persistent activated state that is no longer inhibited by propranolol. However, addition of propranolol at any time prior to complete activation of the enzyme results in inhibition of the catecholamine effect. Paradoxically, addition of catecholamines to the fully activated enzyme that has been washed free of bound catecholamines results in inhibition of enzyme activity. The apparent affinity of catecholamines for the inhibitory process is the same as that of the activating process suggesting that the receptor is not modified in the activated state and probably remains coupled to the enzyme system. Based on these observations, it is suggested that catecholamines serve to increase the rate at which Gpp(NH)p converts the enzyme to its fully activated state. Once attained, it appears that Gpp(NH)p remains tightly bound to this state which is in very slow equilibrium with another, less active, state to which the nucleotide is less tightly bound. These findings also infer that activation of the enzyme occurs via two routes, one mediated by both hormones and guanine nucleotides, the other via guanine nucleotides alone. The latter is a slow, irreversible process (with Gpp(NH)p), the former is a rapid and reversible process.

III. Structure-activity relationships for guanine nucleotides: The structural requirements for the binding and actions of guanine nucleotides at the nucleotide regulatory site have been investigated. Action requires three phosphate groups at the 5'-position of ribose (or 2' and 3' deoxyribose); phosphate groups can be bridged by methylene or imido groups, and a thiol group can be attached to the terminal phosphate. Substitutions on the ribose group or attachment of sugars to the phosphate groups results in reduction or loss of both binding and action of these derivatives. GDP binds to the nucleotide regulatory site with approximately the same affinity as GTP or Gpp(NH)p.

However, GDP does not activate adenylate cyclase and, indeed, inhibits the actions of glucagon and competitively inhibits the actions of Gpp(NH)p or GTP. These findings provide strong evidence for the importance of the nucleotide regulatory site in the activation of the enzyme and its response to hormones.

IV. The fate of GTP and Gpp(NH)p at the nucleotide regulatory site: Studies of the binding of labeled Gpp(NH)p to hepatic and fat cell plasma membranes indicate that the nucleotide binds to sites specific for guanine nucleotides; GTP and GDP inhibit the binding of Gpp(NH)p in a competitive fashion. However, Gpp(NH)p, unlike the naturally occurring nucleotides, binds to the membranes in an essentially irreversible fashion consistent with the findings that adenylate cyclase in these systems is activated by Gpp(NH)p to yield a high activity state that persists even after extensive washing of the membranes to remove unbound nucleotide. Using ^{3}H-GTP and ^{3}H-GDP to examine the fate of these nucleotides when incubated with fat cell and hepatic membranes, it was found that GTP binds rapidly to the nucleotide regulatory site and is converted to GDP; GMP also is detected at the nucleotide binding site. These findings suggest that the different effectiveness of GTP and Gpp(NH)p as activators in the absence of hormones relates to the susceptibility of GTP to hydrolysis at the nucleotide regulatory site. It seems likely, therefore, that the nucleotide regulatory site contains sites for hydrolysis of GTP (and possibly GDP). Since GTP is thought to be the natural regulatory nucleotide for activation of adenylate cyclase in the cell, hydrolysis of GTP at the regulatory site would demand continuous presence of adequate concentrations of GTP for activation of adenylate cyclase; GDP, which also binds to the site and inhibits enzyme activity, may also serve a regulatory role on the system.

V. Computer analysis and model fitting of steady state and transient kinetics of adenylate cyclase. Gpp(NH)p activates all adenylate cyclase systems examined by a time dependent or transient process. The transient phase of activation persists even with saturating concentrations of nucleotide suggesting that the systems exists in at least three states

Gpp(NH)p L
E E' E"
fast slow

which are characterized by a rapid binding phase to form an intermediate state (E'), followed by a slow rate of transition (L) to state E", the high activity state of the enzyme. Hormones abolish the transient phase of Gpp(NH)p activation without altering the final steady state rate achieved by Gpp(NH)p alone. With the aid of a computer fitting and modeling program we examined the kinetic parameters affecting the activity of each of the three states. The kinetic parameters examined were the Km for ATPMg, the productive form of the substrate the K_i for HATP^{3-}, which binds to the catalytic site without being converted to cyclic AMP (and thus is an inhibitor), the K_i for free Mg^{2+} (which theoretically can bind to the catalytic site and compete for ATPMg), and the V_{max}. Kinetic analysis was carried out as a function of ATP, Mg, and HATP^{3-} concentrations (a function of pH, [Mg], and [ATP]) and in the absence or presence of Gpp(NH)p and hormones (glucagon on the hepatic system and ACTH on the adrenal cyclase system). Excellent fits were obtained with the three state model postulated above; each state gave well defined kinetic parameters with respect to K_m, K_i, and V_{max}. With respect to the basis of the transient kinetics of Gpp(NH)p activation, the most interesting state was E' which exhibited a very high affinity for protonated substrate ($HATP^{3-}$) relative to E and E" in both the adrenal and hepatic systems. In addition the adrenal system exhibited a higher affinity for both Mg and ATPMg, and a low V_{max}; i.e.. the intermediate transient state E' differs in the two systems examined. States E and E", by contrast, were virtually identical for both systems. These findings indicated that the adrenal and hepatic system differ not only with respect to the nature of the hormone receptors coupled to the system but also with respect to the presumed structural differences in the transition states leading to the activated state of the enzyme. In all other respects, however, the enzymes behave in essentially identical fashion with respect to their requirements for two regulatory ligands, hormone and guanine nucleotide, to achieve the activated state. The overall activation process regulated by hormone and nucleotide involves the transformation of the enzyme from a state having a high affinity for protonated substrate (and thus an inhibited state) to a state having a considerably lower affinity for protonated substrate and a higher catalytic efficiency.

VI. Effects of guanine nucleotides on fat cell adenylate cyclase. Previous studies showed that GTP stimulates fat cell adenylate cyclase activity in the presence of the various hormones (glucagon, secretin, ACTH, and catecholamines) operating on this multi-receptor system, but paradoxically inhibits enzyme activity by a transient process that is followed by marked stimulation of enzyme activity. As noted above for the hepatic and adrenal cyclase systems, hormones abolish the transient inhibitory phase of Gpp(NH)p action; i.e., they accelerate the rate of transition of the enzyme to its fully active state. Transient inhibition is abolished by increasing the concentration of Mg and pH, conditions which reduce the concentration of protonated substrate, a potent inhibitor of the fat cell enzyme. The inhibitory effect of GTP observed in the absence of hormones is also abolished by reducing the concentration of protonated substrate. Thus, as predicted by the three state transition model described above, GTP and Gpp(NH)p induce the formation of a transition state (E') that is highly susceptible to inhibition by protonated substrate. The complex and seemingly paradoxical behavior of the multi-receptor fat cell system has thus provided strong supportive evidence for the model. However, it seems that the intermediate transition state E' in the fat cell differs from the corresponding state in the adrenal and hepatic system as evidenced by the marked inhibition of enzyme activity induced by the guanine nucleotides on this system. It is possible that this difference is due to a more complex subunit structure of the fat cell system consistent with the fact that this system, in contrast to the other systems examined, contains receptors for at least five different hormones.

VII. Actions of hydroxybenzylpindolol (HBP) on fat cell adenylate cyclase. Pindolol is a potent beta-blocker of catecholamine-sensitive adenylate cyclase systems. It has been shown that HBP also competitively inhibits the actions of catecholamines on the turkey erythrocyte system with an affinity three orders of magnitude higher than that of the catecholamines. Studies of the actions of pindolol and HBP on fat cell cyclase gave rise to the paradoxical finding that HBP is an activator of this system with a 25-fold higher affinity than catecholamines. Pindolol is a weak agonist on this system. Propranolol, a classical beta blocker, only acts as an antagonist. Hydroxybenylpropranolol is a weak agonist but binds to the beta receptor with the same affinity as does HBP. These findings indicate that a catechol group is not essential for activation of adenylate cyclase; a reclassification of the structure functional requirements of the beta receptor seems in order.

Significance to Biomedical Research and the Program of the Institute: A satisfactory model has been developed to explain the complex effects of hormones, guanine nucleotides, ATP, Mg, and pH on the membrane bound adenylate cyclase system in three cell types. Most importantly, the three state transition model provides new insights into the manner by which the enzyme system may be regulated in the target cell for those hormones operating on adenylate cyclase systems. It is now clear that hormones regulate, through their binding and action at the receptors on the external face of the plasma membrane, the actions of guanine nucleotides presumably at the internal side of the membrane. The necessity for two regulatory ligands for conversion of enzyme to its activated state raises the possibility that other membrane bound regulatory processes may similarly require more than one regulatory ligand for control. Finally, the evidence that the adenylate cyclase system takes different transition states having unique kinetic and presumably conformational properties suggests that future studies on these systems must focus attention on the structures of these states before a molecular and physiological basis of hormone action can be understood.

Proposed Course: Continued efforts are being made to solubilize and characterize the component of adenylate cyclase systems. Since Gpp(NH)p activates solubilized preparations of adenylate cyclase even though hormone response is lost, we have the means of at least testing whether the guanine nucleotide regulatory site resides on adenylate cyclase or on a separate component. Once purification of this component is achieved, we can then test whether the regulatory component contains distinct sites for binding and hydrolysis of GTP, the natural regulatory ligand. The purified components will then be used to reconstruct a system that interacts with hormone receptors and which may then become sensitive to the actions of hormones.

Keyword Descriptors:	Adenylate cyclase; hormone action; receptors; guanine; nuclotides; cyclic AMP; plasma membranes; transient kinetics.
Honors and Awards:	NONE

Publications:

Salomon, Y., Londos, C., Rodbell, M.: A highly sensitive adenylate cyclase assay. Anal. Biochem. 58: 541-548, 1974.

Londos, C., Salomon, Y., Lin, M. C., Harwood, J., Schramm, M., Wolff, J., and Rodbell, M.: 5'-Guanylylimidodiphosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. Proc. Nat. Acad. Sci. 71: 3087-3090, 1974.

Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M., and Berman, M.: The role of adenine and guanine nucleotides in the activity and response of adenylate cyclase systems to hormones: Evidence for multiple site transition states. Acta Endocrinol. Supplementum 191: 11-37, 1974.

Salomon, Y., Lin, M. C., Londos, C., Rendell, M., and Rodbell, M.: The hepatic adenylate cyclase system: Evidence for transition states and structural requirements for guanine nucleotide action. J. Biol. Chem. (in press).

Rodbell, M.: On the mechanism of activation of fat cell adenylate cyclase by guanine nucleotides: An explanation for the biphasic inhibitory and stimulatory effects of the nucleotides and the role of hormones. J. Biol. Chem. (in press).

Rodbell, M., Lin, M. C., Salomon, Y., Londos, C., Harwood, J. P., Martin, B. R., Rendell, M., and Berman, M.: Role of adenine and guanine nucleotides in the activity and response of adenylate cyclase systems to hormones: evidence for multisite transition states. In Drummon, G. I., Greengard, P., and Robison, G.A. (Eds.): Advances in Cyclic Nucleotide Research . New York, Raven Press, 1975, pp 3-29.

Rodbell, M., Krishna, G.: Preparation of isolated fat cells and fat cell "ghosts": Methods for assaying adenylate cycalse activity and levels of cyclic AMP. Meth. in Enzym. 31: 103-114, 1974.

Rodbell, M.: Intermolecular coupling in the receptor-adenylate cyclase linked modulation of hormone and transmitter action: The adenylate cyclase system in eukaryotic cells. In Schmitt, F. O., Schneider, D. M., and Crothers, D. M., (Eds.): Functional Linkages in Biomolecular Systems. New York, Raven Press, 1975, pp. 203-213.

Lin, M. C., Salomon, Y., Rendell, M. Rodbell, M.: The hepatic adenylate cyclase system: substrate binding and utilization and the effects of magnesium ion and pH. J. Biol. Chem. (in press).

Rendell, M., Salomon, Y., Lin, M. C., Rodbell, M. and Berman, M.: The hepatic adenylate cyclase system: A mathematical model for the steady state kinetics of catalysis and nucleotide regulation. J. Biol. Chem. (in press).

Salomon Y. and Rodbell, M.: Evidence for specific binding sites for guanine nucleotides in adipocyte and hepatocyte plasma membranes: A difference in fate of GTP and guanlyl-5'yl imidodiphosphate. J. Biol. Chem. (in press).

Schramm, M., and Rodbell, M.: A persistent active state of the adenylate cyclase system produced by the combined actions of isoproterenol and guanylyl-imidodiphosphate in frog erythrocyte membranes. J. Biol. Chem. 250: 2232-2237, 1975.

Project No. Z01 AM-15001-01 LNE
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure-function relationships for glucagon and secretin.

Previous Serial Number: None

Principal Investigator: Dr. Michael C. Lin

Other Investigators: Dr. Victor J. Hruby and Dr. David E. Wright, University of Arizona, Tucson.

Cooperating Units: None

Man Years:

Total:	0.50
Professional:	0.50
Other:	0

Project Description:

Objectives: The ultimate goal of this study is to determine the configuration of glucagon required for binding and action at its receptor. Some knowledge can be gained by chemically modifying or deleting various amino acid residues in the molecule and determining whether such changes result in alterations of the binding and actions of the hormone.

Methods: Des-His^1-glucagon was prepared by an insoluble Edman reagent followed by purification with column chromatography. Des-(Asn^{28},Thr^{29})-homoserinelactone^{27}-glucagon was prepared by CNBr treatment and purified by partition chromatography. The purity of these derivatives was examined by amino acid analyses. The biological activity of modified glucagon was determined by their ability to activate the adenylate cyclase system in liver. The affinity was measured by their specific binding to the hepatic plasma membrane. Both solid phase and classical methods have been used for chemical synthesis of secretin.

Major Findings: (Asn^{28},Thr^{29})-homoserinelactone^{27}-glucagon lost both affinity and biological activity equally by 50-fold. In contrast, des-His^1-glucagon displayed a 15-fold decrease in affinity but a 50-fold decrease in activity relative to that of native glucagon. Therefore, the role of two hydrophilic residues at the COOH-end is strictly in the recognition process while NH_2 - terminal histidine plays an important role in the expression of hormone action. The chemical synthesis of secretin is approaching completion.

Significance to Bio-Medical Research and the Program of the Institute: The knowledge of the structural requirement of hormone for its function is fundamental to the understanding of hormone action. In view of the recent discovery that the development of severe diabetic hyperglycemia requires the presence of glucagon, a modified glucagon with antagonistic property may have potential therapeutic value.

Proposed Course of Project: Chemical modification of glucagon will be continued. In particular, chemical alteration in the degree of hydrophobicity of glucagon will be studied closely in terms of its effect on the affinity and biological activity of the hormone. Synthetic secretin will be ready for testing before the end of 1975. The availability of this hormone will enable us to study its action closely with the same approach that we applied successfully to the study of glucagon.

Keyword Descriptors: Adenylate cyclase; glucagon; receptor.

Honors and Awards: NONE

Publications:

Lin, M. C., Wright, D. E., Hruby, V. J., and Rodbell, M.: Structure-function relationships in glucagon: Properties of highly purified des-His^{1}-monoiodo- and des-Asn^{28}, Thr^{29}, homoserinelactone^{27}-glucagons. Biochemistry 14: 1559-1563, 1975.

Chan, P. S., and Lin, M. C.: Isolation of cyclic AMP by inorganic salt coprecipitation. Meth. in Enzym. 38: 28-41, 1974.

Lin, M. C.: An automated chromatographic assay for adenylate cyclase and cyclic 3',5'-AMP phosphodiesterase. Meth. in Enzym. 28: 125-135, 1974.

Lin, M. C.: An allosteric model for glucagon activation of hepatic adenylate cyclase system. In Kahan, B. D. and Reisfeld, R. A. (Eds.): The Cell Surface. New York, Plenum Press, 1974, pp. 232-237.

Project No. Z01 AM-15002-01 LNE
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Binding of unlabeled glucagon to rat liver membranes.

Previous Serial Number: NONE

Principal Investigator: Dr. Constantine Londos

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	.3
Professional:	.3
Other:	0

Project Description:

Objectives: Three aspects of the interaction of glucagon with rat liver plasma membranes have been described previously: 1) binding of ^{125}I-glucagon, 2) activation of a glucagon-specific adenylate cyclase system, 3) inactivation of hormone by a "glucagon-specific inactivating mechanism" which destroys glucagon at low (nanomolar) concentrations. As can be seen, the inactivating phenomenon complicates any effort to quantitate phenomena 1 and 2, above. If, however, the inactivation phenomenon were shown to be an artifact arising from the use of ^{125}I-glucagon, which may differ from the native molecule in its reaction with the hormone receptor, quantitative information on the binding of glucagon should be obtainable. To this end, studies on the binding of native, unlabeled glucagon to hepatic membranes are conducted.

Methods: Binding of unlabeled glucagon to liver membranes was determined by incubating hormone with liver plasma membranes, followed by centrifugation of membranes and measuring glucagon remaining in the supernatant. Glucagon was assayed with the use of the hepatic adenylate cyclase system. Inclusion of GTP in the adenylate cyclase assay medium which reduces the concentration of glucagon required for half-maximal activation of the enzyme, permitted detection of low levels of glucagon in aliquots of the supernatants from the binding phase of the experiment.

The time course of glucagon uptake was followed by filtering the binding reaction mixture in a Swinny device, and the glucagon in the filtrate determined as described above.

Major Findings: These studies have revealed that the purported inactivating mechanism does not exist, and that there are differences between the binding of native and radioiodinated glucagon to membranes. The removal from the binding medium of native glucagon by membranes is much greater than that of ^{125}I-glucagon. However, much of the "lost glucagon is found bound to the membranes and may be released upon mild sonication at 55° C. (While liver membranes do contain enzymes which destroy glucagon, inclusion of bacitracin in the medium blocks the actions of such enzymes). Moreover, the rate of equilibration of the native hormone with the membranes, as determined by Swinney filtration experiments, is considerably greater than the rate observed with the labeled molecule. The problem with the labeled glucagon appears not to be related to the iodination reaction or to the presence of the iodine atom(s) in the molecule, as I-glucagon and glucagon are indistiguishable in their effects on the hepatic adenylate cyclase. It is likely that the beta radiation of the radioiodine damages the hormone, leading to reduced binding.

The failure to find any detectable loss of glucagon from the binding medium in the presence of adrenal membranes or heat-treated liver membranes indicates that non-specific absorption of hormone to membranes does not account for the observed binding. More importantly, guanine nucleotides, such as GppNHp, which play a central role in the regulation of adenylate cyclase systems, were found to decrease considerably the binding of the native hormone. The latter agrees with earlier findings obtained with ^{125}I-glucagon. Finally, earlier published studies on the "inactivating" phenomenon established that the process was specific for glucagon. Although the present studies show that the hormone is actually bound, and not inactivated, the criteria by which the specificity of the phenomenon was established would still apply.

Studies on the kinetic characteristics of glucagon binding, still in progress, indicate that the affinity for the native hormone is slightly greater than for the labeled hormone. Total binding capacity for native glucagon, 10^{-15} pmoles per mg membrane protein, is greater than that for labeled hormone, 2-3 pmol per mg membrane protein.

Significance: The relationship in plasma membranes between hormone receptors and the catalytic components of adenylate cyclase systems (or any other hormone-responsive system) remains a mystery. A necessary first step in defining this relationship is to determine the number of receptors, a question yet unanswered in view of the problems associated with the use of ^{125}-I-glucagon, but which should yield to analysis with the native hormone.

The methods employed in these studies for investigating glucagon binding are tedious and would be impractical, or in some case, impossible, for examining binding at very low hormone concentrations or in the presence of guanine nucleotides. They have, however, revealed the problems one may encounter with the use of ^{125}-I-glucagon, and do provide a standard for determining the efficacy of any other labeled glucagon preparations. In view of the recently discovered role of glucagon in the hyperglycemia of the diabetes syndrome, and with the expectation that studies on the actions of glucagon will be intensified, the importance of such information is obvious.

Proposed Course of Project: The course of these studies shall be limited to an accurate determination of the affinity of liver membranes for glucagon and to a determination of the number of hormone binding sites.

Keyword Descriptors:	Glucagon binding; glucagon inactivation, iodinated glucagon.
Honors and Awards:	None
Publications:	None

Project No. Z01 AM-15003-01 LNE
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Analysis of adrenal adenylate cyclase.

Previous Serial Number: NONE

Principal Investigator: Dr. Constantine Londos

Other Investigators: Dr. Marc Rendell

Cooperating Units: Laboratory of Theoretical Biology, National Cancer Institute

Man Years:

Total:	.4
Professional:	.4
Other:	0

Project Description:

Objectives; While numerous adenylate cyclase systems have been studied intensively, there have been few efforts to characterize the ACTH-sensitive adrenal enzyme system. We investigated the rat adrenal adenylate cyclase system for purposes of comparison with other adenylate cyclase systems being studied in this laboratory. The adrenal enzyme is unique in that the hormone response is selectively inhibited by the chelator, EGTA. The latter may provide an additional tool for defining the relationship between receptor and catalytic components of the enzyme system. Moreover, adrenal tumor cells which lack functional ACTH receptors are available for study.

Major Findings: In the presence of low substrate concentrations (0.1 mM), activation of the adrenal enzyme by ACTH is absolutely dependent upon guanine nucleotide (GTP or GppNHp) at low concentrations, and the actions of hormone and nucleotides are synergistic, GDP, on the other hand, inhibits activity and blocks the action of GTP and GppNHp, revealing that the terminal phosphate of the guanine nucleotide is required but need not be hydrolyzed in the activation process.

Extensive kinetic analysis of the adrenal enzyme had revealed the following. The enzyme system conforms to the three state model developed for the hepatic glucagon-sensitive adenylate cyclase system. The three defined activity states differ in their affinity for substrate and in their susceptibility to inhibition by magnesium and protonated substrate. However, the adrenal enzyme is unique in that in the activated states magnesium competes with the substrate, MgATP, at the catalytic site. That no such inhibition was observed with the hepatic enzyme indicates that adenylate cyclase systems may differ not only at their receptor components but at their catalytic components as well.

A further finding was that the "basal" activity, i.e that measured in the absence of hormone and guanine nucleotide, reflects a mixture of enzyme states, and that the proportion of enzyme in any given state varies from preparation to preparation. Enzymes having a high proportion of the activated states could be reverted to states of lower activity *in vitro*.

Significance to Bio-Medical Research and the Program of the Institute: Given the acknowledged role of cyclic AMP in the metabolic scheme of most cells, the regulation of the enzyme which produces this important compound is evident. These studies have further defined the requirements of the guanine nucleotide-sensitive regulatory component of the adrenal enzyme. These studies also question current dogma that adenylate cyclase systems are homogeneous with respect to their catalytic components, differing only in their receptor components. Finally, the "basal" state of the enzyme is often investigated in disease states to determine if alteration of adenylate cyclase is the cause of the disease. Our studies indicate that the "basal" enzyme reflects a mixture of enzyme states, meaning that alterations of the enzyme in disease may reflect the metabolic state of the cell and not an alteration of the enzyme *per se*.

Proposed Course of Project: The mechanism of inhibition of the ACTH response by chelators will be investigated. It has been proposed that calcium is required to complement the hormone signal for adenylate cyclase activation. However, our preliminary data suggest that EGTA mimics the action of protonated substrate, and that either of these two compounds prevent the transition to the activated state of the enzyme. Such studies may reveal the presence of a functionally important metal ion in the enzyme.

Keyword Descriptors: Adrenal adenylate cyclase; Kinetic analysis.

Honors and Awards: NONE

Publications:

Londos, C., and Rodbell, M.: Multiple inhibitory and activating effects of nucleotides and magnesium on adrenal adenylate cyclase. J. Biol. Chem. 250: 3459-3465, 1975.

Project No. Z01 AM-15100-05 LNE
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, MD 20014

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein-Nucleic acid interactions: Chromatin structure and function.

Previous Serial Number: NIAMDD-LNE-11

Principal Investigator: Dr. Robert T. Simpson

Other Investigators: Dr. James P. Whitlock, Ms. Lois Tack and Ms. Linda Propst.

Cooperating Units: None

Man Years:

Total:	2.5
Professional:	1.25
Other:	1.25

Project Description:

Objectives: To study the structural and enzymatic bases for the restriction of translation of the major portion of genetic information in nucleated, mammalian cells; and to study the mechanisms which allow transcription of that portion of the genetic message which is appropriate to the function of the particular cell type.

Methods Employed: Cells in tissue culture (HeLa, S-91 melanoma, or cloned myoblasts) are grown in the presence of dBrU, or dIU, with or without dFU, to substitute all or part of the thymidine residues in DNA with an analogue. Chromatin is also isolated as 11S subunits by brief micrococcal nuclease digestion of liver nuclei, hypotonic rupture and sucrose gradient sedimentation. RNA is isolated from cells or nuclei, poly A containing RNA by interaction with polydeoxythymidylate-cellulose, and poly- and oligo-A segments by specific nuclease treatment. RNA is characterized by size distribution on sucrose gradient sedimentation and polyacrylamide gel electrophoresis.

Physicochemical properties of the 11S particles are determined in standard fashion. Heterogeneity of the particles is judged by analytical and preparative isoelectric focusing followed by histone isolation and analysis. Whole chromatin and 11S particles are reacted with acetic anhydride, histones isolated and digested with proteolytic enzymes. The digested peptides are fractionated by chromatographic and electrophoretic techniques, isolated peptides examined for acetate content and amino acid composition by radioactivity measurments and analysis using a specially modified amino acid analyzer currently operating at the 50 picomole level.

Major Findings: The replacement of thymidine by its analog dBrU, in the DNA of proliferating cells is associated with phenotypic changes, e.g., alterations in specific protein synthesis, changes in enzyme inducibility, in several cell types. Cell viability is apparently unaffected. The mechanism of this selective effect on "differentiation" is unknown. We have previously characterized the chromatin of HeLa cells extensively substituted with dBrU and now we are studying nuclear RNA metabolism in these cells grown for one generation in dBrU to evaluate what effects of the analog occur at the transcriptional level.

The apparent rate of RNA synthesis is significantly lower (ca. 50%) in dBrU cells when compared to controls, although with time (2 hr) this difference is largely erased. The rate of synthesis of nuclear RNA containing polyA sequences is similarly altered by the presence of the thymidine analog. In contrast to these differences in rate of synthesis, the size distribution of both total rapidly labeled nuclear RNA and that fraction which contains polyA sequences is indistinguishable for the control and dBrU containing cells. The synthesis of both the long 3' polyA tails and the shorter internal oligoA regions of nuclear RNA appears unaffected by the presence of the thymidine analog. It thus appears that, barring major differences in pool sizes (currently under investigation), dBrU induces a quantitative defect in synthesis of nuclear RNA. The basis for this effect, i.e., initiation vs propagation, and attempts to investigate qualitative alterations in the synthesis of specific RNA species will constitute further approaches to using dBrU as a probe for control of differentiated functions in eukaroytic cells.

We had shown earlier that acetylation of native chromatin with acetic anhydride led to modification of a limited number of the lysyl residues of the histones, presumably those which are not bound to DNA in the nucleoprotein complex. Histones from such chromatin, labeled with radioactive acetate, are extracted, fully cold acetylated with acetic anhydride, and then digested with trypsin to yield the peptides derived by arginyl cleavages. The digestion is complete and over 90% of the label is soluble in 1% acetic acid after digestion. On two-dimensional fingerprints of the digest, about 65 spots are routinely detected with a fluorescamine spray, about 5-10 more than theoretically predicted. Counting the spots after cutting from the paper reveals that the bulk of the radioactive label is confined to some 9-11 peptides which move slowly on both butanol-acetic acid-water chromatography and electrophoresis at pH 3.5. Chromatography of the digest on Sephadex G-25 in 1% acetic acid demonstrates that the bulk of the label is associated with large peptides, excluded or nearly excluded from this column medium, while the bulk of the peptides, measured by fluorescamine assay, are small peptides, eluting near the salt volume of the column. Fractionation by G-25 followed by two-dimensional fingerprinting will apparently allow isolation of the labeled peptides of interest without prior fractionation of the individual histones, thus circumventing the difficulties we have had in the past with isolation of pure modified individual histones. We hope that sufficient material can be eluted from the paper to allow identification of the peptides in the known primary sequences of the individual histones. We estimate that even a 10% yield of peptide from paper will give about 50 nanomoles of peptide. Since Dr. Robert Hartley's modified amino acid analyzer is currently operating at the 50 picomole level, we should be able to achieve our goal of peptide identification without having to develop column peptide fractionation methodology.

In the past year there has been developed a body of evidence suggesting that the bulk of chromatin is organized in vivo as a string of repeating subunit structures, each probably composed of a multimer of eight histone molecules with an associated piece of DNA ca. 200 base pairs in length. Noll has reported the isolation of such particles from rat liver nuclei after brief nuclease digestion. We have reproduced his results and now isolate 11S particles after such digestion by sucrose gradient sedimentation. Our initial efforts to use these chromatin subunit particles to study chromosome structure have revolved around attempts to see if all 11S particles were the same, that is each contained 2 molecules each of the four smaller histones, or if they differed, some containing f2a1 and f3 and others f2b and f2a2 histones, for example. Separation was attempted by ion-exchange chromatography on ECTHAM-cellulose and by preparative isoelectric focusing in sucrose gradients, both in the presence and absence of urea concentrations up to 6 M.

By neither method can we detect any heterogeneity in the makeup of the 11S particles. Further, analytical measurements of the content of the various histones across either the column elution profile or the focusing peak indicate that the same amount of each of the four histones is present in each fraction examined. Thus, 11S particles appear to comprise a single species and are appropriate for physical study without further fractionation.

Significance to Bio-Medical Research and the Program of the Institute: Determination of the structure of the eukaryotic nucleoprotein complex, chromatin, is necessary for understanding the mechanism of gene regulation in higher organisms. When we can describe in detail the binding of histones to DNA in chromatin, the goal of the acetylation experiments, we will be able to generate meaningful models for protein-DNA interactions in this complex. Study of the molecular consequences of dBrU substitution should allow correlation of altered structure and function of chromatin with altered development of a cell in vivo, furthering our efforts to understand the details of gene egulation in normal and diseased cells.

Keyword Descriptors: Chromatin; 5-deoxybromouridine; gene regulation, 11S particles; peptides; chemical modification; histones.

Honors and Awards: NONE

Publications:

Simpson, R. T. and Seale, R. L.: Characterization of chromatin extensively substituted with 5-bromodeoxyuridine. Biochemistry 13: 4609-4616, 1974.

Piatigorsky, J., Zelenka, P. and Simpson, R.T.: Molecular weight and sub-unit structure of delta-crystallin from embryonic chick lens fibers. Exp. Eye Res. 18: 435-446, 1974.

Project No. Z01 AM-15101-04 LNE
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Analysis of the active and inactive portions of the eukaryotic genome.

Previous Serial Number: NIAMDD-LNE-13 and NIAMDD-LNE-15

Principal Investigator: Dr. Robert Simpson

Other Investigators: Ms. Linda Propst and Dr. Gerald Reeck

Cooperating Units: None

Man Years:

Total:	1.25
Professional:	0.75
Other:	0.50

Project Description:

Objectives; To determine the physico-chemical basis for the functional differences between the portions of the eukaryotic genome which are transcribable *in vitro* and the remainder of the chromosome.

Methods Employed: Chromatin is isolated by conventional methods, sonicated to a size of *ca.* 1000 DNA base pairs, and fractionated by ion-exchange chromatography on the weak anion exchanger ECTHAM-cellulose. Physicochemical characterization of the fractions thus isolated is carried out using standard techniques, including specifically, measurement of binding of ethidium bromide by fluorescence titration, determination of DNA molecular weights by classical sedimentation equilibrium methodology, measurement of reannealing rates by hydroxyapatite chromatography, and isopycnic banding of DNA in CsCl solutions.

The "subunit" structure of chromatin is investigated by digestion for varying periods of time of the isolated chromatin with DNase II at pH 7.0 and 37°. Digestion mixtures are analyzed by sedimentation on linear 5-20% sucrose gradients and measurement of DNA distribution in the gradient spectrophotometrically. Sedimentation constants are calculated assuming a linear change in solvent viscosity and density from meniscus to peak position.

Rabbit liver polysomes are isolated by methods described by Palmiter using magnesium precipitation. Polysomal RNA is isolated by phenol-chloroform extraction and the polyA containing RNA isolated from the bulk by adsorption to polyuridylate-Sepharose in high salt and elution with water at 23°. A cDNA complementary to this RNA was prepared by Dr. Judy Kantor, NHLI. The DNA was purified from nonacid precipitable contaminants by Sephadex G25 chromatography and has a sedimentation constant of 9-10 S. Hybridization reactions are carried out under standard conditions and analyzed by hydroxyapatite chromatography.

Nonhistone chromosomal proteins are isolated from dehistonized chromatin by the DNase I digestion procedure of Wagner and Spelsberg and analyzed on 28 cm long discontinuous slab gels containing a gradient of 5-15% polyacrylamide.

Major Findings: Two types of binding of ethidium bromide have been observed for whole chromatin, differing in number of sites and stability. Repressed chromatin also has both types of binding sites with stabilities and stoichiometries similar to those observed by others. In contrast, chromatin eluted late from ECTHAM-cellulose (transcribable chromatin) lacks the tight binding class of sites. Further experimentation to define the nature of the two classes of sites will be required to allow interpretation of these data in terms of their implications for the structure of the transcribable portions of the genome.

Satellite DNA is known to be associated with the constitutive heterochromatin of animal cells, a type of chromatin which is not transcribed, remains heteropycknotic throughout the life cycle of the cell, and probably serves a structural role, being concentrated near centromeric regions of the metaphase chromosome. Using mouse liver as a tissue source, we have investigated the distribution of satellite DNA in ECTHAM-cellulose fractions. Due to the small size and consequent broad banding of our DNA, it was necessary to first dissociate and reanneal to a C_0t of 0.01 - 0.1. This both allows the satellite sequences to reanneal and form networks of higher molecular weight, and hence band sharply at a density of 1.690 and, by keeping the non-satellite sequences in single stranded form, increases their isopycnic density to 1.715 and facilitates satellite detection by increasing the density difference with other forms of DNA.

The content of satellite DNA in our chromatin preparations, 8%, is similar to that in whole mouse liver nuclear DNA. The earliest fractions eluted from ECTHAM-cellulose columns have an augmented content of satellite, usually in the range of 17-21%. In contrast, the transcribable fractions eluted last from the column nearly lack satellite DNA sequences, values of 1-3% being observed in a number of experiments. These data are supported by the portion of the DNA which is double stranded after annealing to a C_0t of 0.02. The earliest fractions have about 18% of their sequences annealed at this point while the last eluted material has only about 4%. It is apparent that constitutive heterochromatin is the earliest eluted species on ECTHAM-cellulose chromatography of sonicated chromatin.

DNase II digestion of chromatin generates a spectrum of particle sizes, ranging from 11 to over 20 S, which are gradually converted to only 11 S species. Measurements of DNA lengths in the particles by Oosterhof, Hozier and Rill suggested that they comprise various multimers (monomer, dimer, trimer, etc), of the basic repeating subunit particle which seems to be characteristic of all eukaryotic chromatins. We have used this technique to see if the chromatin fractions we obtain by ECTHAM-cellulose chromatography differ in their subunit composition. Repressed chromatin fractions yield the same spectrum of multimers yielded by whole chromatin with a similar time course of digestion. In contrast, fractions enriched in constitutive heterochromatin give a lower yield of particles, some 40% of the chromatin DNA being undigested even after 7 hours incubation at 37^{o}. Isolation of the DNA from this undigested material reveals it to consist of ca. 50% satellite DNA sequences. Hence we conclude that constitutive heterochromatin differs fundamentally in its structure from the beaded chain model proposed for the bulk of chromatin. We also find differences in examining the subunit composition of the transcribable chromatin isolated by ion-exchange chromatography. In this case, however, we find a faster rate of digestion and further, we see essentially only monomeric subunits. Few dimers and no higher orders of multimers are observed. From this observation we suggest that the transcribable chromatin must have relatively longer stretches of free DNA between single histone beaded subunits.

This contention was investigated by determination of the length of the segments of the DNA of chromatin fractions which was susceptible to digestion by micrococcal nuclease. Such segments are isolatable by sequential treatment of chromatin with poly-D-lysine, pronase, micrococcal nuclease, and chloroform-isoamyl alcohol extraction. The susceptible segments of the earliest-eluted chromatin were ca. 45 base pairs long, those in the middle region of the elution profile about 130 base pairs, while those associated with the transcribable chromatin were over 200 base pairs in length. Thus, while the beaded subunit model for chromatin probably accurately reflects the structure of the bulk of the genome, there are significant differences from this general pattern for at least two subspecies of chromatin, constitutive heterochromatin and that portion of chromatin which is readily transcribable in vitro.

While we feel that we have clearly demonstrated that the fractionation procedure which we employ separates segments of chromatin which are repressed from those which are readily transcribable in vitro, it remains to be established if these latter segments also contain the genetic regions which are transcribed in vivo. To approach this question we have examined the hybridization of cDNA's to the DNA of ECTHAM-cellulose chromatin fractions. The chromatin source is always rabbit liver. Using polysomal polyA-containing RNA from rabbit liver, the cDNA should localize turned on gene sequences. Using globin cDNA (a gift from Dr. French Anderson, NHLI) should localize turned off gene sequences. While there have been a number of technical difficulties in moving this project, we feel that they have now been solved and we have obtained preliminary information for the turned off system. Relative to total rabbit liver DNA, globin gene frequencies for five ECTHAM-cellulose fractions were determined as follows from the maximal level of hybridization observed: Early - 0.4, early-middle - 1.2, middle - 2.1, middle-late - 0.8, and late - 0.3. Such findings are consistent with the sequential elution of three types of chromatin segments from ECTHAM-cellulose columns -- constitutive heterochromatin (depleted in globin genes), repressed euchromatin (enriched in globin genes in rabbit liver chromatin) and transcribable euchromatin (depleted in globin genes in a turned off system). Data for the polysomal polyA-containing RNA are currently not available for the chromatin fractions, but the probe does anneal appropriately to whole rabbit liver DNA.

Significance to Bio-Medical Research and the Program of the Institute: Probably the most direct approach to understanding the gross basis for transcriptional regulation in eukaryotic cells is the study of the composition and structure of separated repressed and transcribed segments of the genome. We have developed methodology which seems to allow separation of these two types of nucleoprotein and our current studies have established major differences in both their structure and composition. Extension of these studies to detect more subtle differences in structure between the chromatin species, *e.g.*, mode of histone binding, should allow still more detailed insight into the mechanism of transcriptional regulation and its alterations in development or in disease states.

Keyword Descriptors: Chromatin, gene regulation; ECTHAM-cellulose chromatography; cDNA; 11 S subunits; satellite DNA; constitutive heterochromatin; mRNA.

Honors and Awards: None

Publications:

Simpson, R. T.: Distribution of RNA polymerase binding sites in fractionated chromatin. *Proc. Nat. Acad. Sci. U.S.A.* *71*, 2740-2743, 1974.

Reeck, G. R., Simpson, R. T., and Sober, H. A.: The distribution of histones and nonhistone proteins in the ECTHAM-cellulose fractions of chromatin from several tissues. *Eur. J. Biochem.* *49*: 407-414, 1974.

Project No. Z01 AM-15102-15 LNE
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure and function of an extracellular ribonuclease (barnase) and its intracellular inhibitor (barstar) from Bacillus amyloliquefaciens.

Previous Serial Number: NIAMDD-LNE-9

Principal Investigator: Dr. Robert W. Hartley

Other Investigators: None

Cooperating Units: Large-scale Laboratory, Office of Chief, LNE, NIAMDD. Collaboration with D. M. Blow, and R. C. Sheppard, (Laboratory of Molecular Biology, Medical Research Council, Cambridge, England).

Man Years

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description

Objectives: To further characterize and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of structure, function and synthesis.

Specific objectives are: (1) the determination of the amino acid sequence and three-dimensional structure (by X-ray diffraction) of both proteins and the structure of their complex; (2) thermodynamic and kinetic analysis of structural transitions produced in both proteins by heat or denaturing reagents; (3) analysis of the folding and activity of both by means of peptide complementation systems analogous to those being used for the study of pancreatic ribonuclease and micrococcal nuclease; (4) all of the above with chemically and/or genetically modified sequences.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several others) are grown in synthetic media. Barnase is adsorbed from the culture medium onto phosphocellulose, barstar is extracted from the cells. Their purifications make use of ion exchange, gel-permeation and affinity chromatography.

Work on the determination of amino-acid sequences involves amino acid analysis, enzymatic digestions, peptide separations and peptide sequencing by dansyl-Edman and difference-Edman techniques. Peptide separation and amino acid analysis are by automated ion-exchange chromatography using a modified JEOLCO analyzer equipped for detection with Roth's orthophthaldehyde reagent. Dansyl amino acids are identified by thin-layer chromatography on polyamide layers.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents are studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, circular dichroism, exclusion chromatography, with the use of proteolytic enzymes and by equilibrium binding studies. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of each peptide alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Crystals of barnase are grown in hanging drops (10 µl) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure has been used to synthesize portions of the barnase sequence.

Major Findings: It has been shown rigorously that the sodium dodecyl sulfate (SDS) induced structural transition of barnase is reversible and "two-state" in nature. That is, at equilibrium virtually all molecules are in the native form, which binds no SDS, or in the SDS-form, which binds 14 ± 1 SDS molecules. Binding experiments using equilibrium dialysis show that above the transition range (> 1mM) binding of SDS increases and is a function of salt concentration. 14 moles of SDS per mole of barnase is equivalent to 0.33 grams/gram, much lower than the 1.4 grams/gram found generally for proteins lacking disulfide bonds at high (> 2mM) SDS concentrations. The number (14) is constant between 0.5 and 0.8 mM SDS, however, and must have structural significance.

A JEOLCO amino acid analyzer has been extensively modified to take advantage of the sensitivity of Roth's fluorescence-producing orthophthaldehyde reagent. In addition to higher sensitivity, this reagent allows a simpler and more reliable detection system than fluorescamine because of its stability in aqueous buffers.

To take full advantage of its sensitivity, pump noise has been eliminated by using tank pressure, with reduction valves, to drive and control all flows. A novel arrangement allows programming in as many as 8 different eluants without their being individually pressurized. The machine is currently being used to analyze samples containing about 50 picomoles per residue. It could easily be used another order of magnitude lower if it were not for the problems of handling such small samples without loss or contamination.

It has recently been found that barnase crystals may be transferred from the $(NH_4)_2SO_4$ solvent in which they are grown to near-saturated $MgSO_4$. They become fragile but, we hope, not too much so. This should greatly increase our chances of obtaining isomorphous crystals containing heavy element compounds, many of which can bind to protein amino groups.

It has not been found possible to achieeve reliable high yields of both barnase and barstar in a single run in the large (1100 liter) fermentor. Satisfactory conditions for good yields of one or the other have been worked out however, and for the present separate production runs are being made for each.

Significance to Bio-Medical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase made it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. The inhibitor is an even simpler protein and should be useful in a similar fashion. Together, they offer a model system for the study of a protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure. The production of both of these proteins by the same organism is also of biological interest.

It has been suggested that protein-detergent interactions may serve as models for interactions between proteins and phospholipids in biological membranes.

Proposed Course of Project: Collaboration with the workers in Cambridge on the X-ray solution of barnase structure and on peptide complementation studies will continue. Sequencing of barstar and attempts to grow crystals of barstar and the complex will continue.

The studies of barnase-detergent interactions will be extended to an investigation of the reaction between barnase and cationic detergents.

Keyword Descriptors: Barnase; barstar; *Bacillus amyloliquefaciens*; ribonuclease; amino-acid sequence; protein conformation.

Honors and Awards: None

Publications;

Hartley, R. W.: A two-state conformational transition of the extracellular ribonuclease of Bacillus amyloliquefaciens (barnase) induced by sodium dodecyl sulfate. Biochemistry (in press).

Project No. Z01 AM-15103-03 LNE
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemistry of pituitary glycoprotein hormones and the role of pituitary proteolytic enzymes in their secretion and activation.

Previous Serial No. NIAMDD-LNE-18

Principal Investigator: Dr. Peter G. Condliffe, LNE, NIAMDD

Other Investigators: Dr. J. A. Gladner, LBP, NIAMDD
Professor Nobuo Ui, Gunma University, Japan

Cooperating Units: Department of Physical Biochemistry, Institute of Endocrinology, Gunma University, Japan.

Man Years

Total:	2.0
Professional:	2.0
Other:	0

Project Description:

Objectives: During the past decade, the sub-units of the pituitary hormones, TSH, LH and FSH have been isolated from human glands and from several species of animal glands. As a result, the peptide sequences are known for the subunits of b-TSH, b-LH, and b-FSH. Partial information from peptide mapping of the human hormones, together with recombination experiments involving their various sub-units, strongly suggest that the structures of h-TSH and h-LH are very close to those of the bovine.

The demonstration in several laboratories that the glycoprotein hormones from the pituitary and also from the placenta, possess 2 sub-units, not linked by disulfide or other covalent bonds, raises several questions about their synthesis and the regulation of their secretion.

1. Are the sub-units, which each have between 100 and 120 amino acid residues, synthesized separately and then combined to yield the active hormone or are they derived from a single pro-hormone as is the case with insulin.

2. If the subunits are synthesized separately, are there, in fact, two pro-hormones each of which is activated and which then are combined to yield the active hormone?

3. Do the pituitary proteolytic enzymes play a role in the secretion and activation of these hormones?

Methods Employed: Against this background, a detailed investigation of the pituitary proteolytic enzymes has been started. Their role in the conversion of putative pro-hormonal forms of the glycoprotein hormone to the active forms found in the pituitary and the circulation is being studied using bovine, salmon and human glands.

Proteolytic activity is assayed by the method of Avron using denatured human hemoglobin prepared from out-dated blood from the NIH blood bank.

The glycoprotein hormones and their derivatives are identified by radioimmunoassay, gel electrophoresis, and their behavior in gel filtration and other techniques.

Results: Bovine and whale TSH, LH and FSH are both adsorbed to Concanavalin A-Sepharose at pH 7 in 0.02 M Tris - 0.2 M NaCl. Binding of TSH and of the other glycoprotein hormones LH and FSH was improved in 1 mM $CaCl_2$. The glycoprotein were eluted with 0.5 M α-methyl-D-glucose or 0.5 M α-methyl-D-mannoside.

This technique was applied to the purification of the glycoprotein hormones. Following extraction from lyophilized glands the hormones were adsorbed to Concanavalin A-Sepharose. 90% of the TSH activity measured by immunoassay is removed from the extract in two passes. FSH and LH were also adsorbed although the efficiency was not tested quantitatively. After washing with 1 M NaCl the hormones were washed with α-methyl-D-glucoside and fractionated by ion exchange chromatography and gel filtration. Two preparations of bovine TSH have been prepared using different procedures. The first was prepared using the affinity techniques described above in combination with cellulosic ion exchangers and gel filtration. The biological activity and immunoreactive properties were the same as for the second preparation that was purified by the old procedures. However the yields were higher. It may be possible to eliminate some rechromatography steps that used to be required.

Whale and bovine TSH and LH were compared using the ANS-fluorescence technique of Edelhoch and collaborators, to follow their disaggregation into their subunits. While the LH preparations behaved as found by Edelhoch, difficulty was encountered with TSH. Only partial disaggregation of b-TSH was obtained under their conditions. This may be due to variations in the carbohydrate position of the glycoproteins.

Some preliminary experiments suggest that disaggregated whale and bovine subunits will cross react under appropriate conditions to yield active whale-bovine hybrid molecules of LH and TSH.

Proposed Course of Project: The action of the pituitary proteases on the sub-units of the hormones will be studied. Differences will be looked for in the proteolysis of the isolated sub-units by standard techniques for study of protein degradation (gel electrophoresis, peptide mapping etc.). The effects of proteolysis on the immunochemical properties of the hormones and the disaggregated sub-units will also be assessed.

Isolation of the pure hormones will be undertaken from crude extracts using affinity chromatography and related techniques. If pituitary protease activity can be successfully inhibited, a search will be made for prohormonal components of the pituitary. By analogy with work on insulins, such components should react with antisera directed against the hormones already isolated. Most partly purified prepartions of b-TSH contain multiple components some of which appear to be of higher molecular weight. During gel filtration of crude TSH fractions on G-100, a small amount of TSH activity is usually unretarded. Also, in past experiments in which b-TSH extracts were rechromatographed on CM-cellulose, about 10% of the biological activity was usually unadsorbed; rechromatography of this fraction, which was of low potency, showed that it differed in many respects from the highly purified TSH fractions that were isolated. These experiments were carried out before the development of radioimmunoassays by Berson and Yalow. At that time (before 1960) these experiments were interpreted as being partly denatured, or aggregated material or possibly as TSH adsorbed to other proteins. With the evolution of current ideas about pro-hormones, these old experiments suggest that pro-hormonal forms of TSH and other glycoprotein hormones are waiting to be found.

Keyword Descriptors: Thyroid stimulating hormones - TSH; Whale-TSH; Bovine-TSH; Affinity chromatography; Concanavalin-A-sepharose.

Honors and Awards: NONE

Publications: NONE

Project No. Z01 AM-15104-03 LNE
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation of large oligomers from viral nucleic acids in preparative (mg) amounts.

Previous Serial Number: NIAMDD-LNE-10

Principal Investigator: George Rushizky

Other Investigators: H. A. Sober (deceased), D. L. Rogerson, Jr., and V. A. Shaternikov, Ministry of Health, Moscow, USSR.

Cooperating Units: Large scale laboratory, LNE-NIAMDD

Man Years:

Total:	2.5
Professional:	1.5
Other:	1.0

Project Description

Objectives: To isolate and/or to develop procedures for the preparation and isolation of large oligonucleotides (fragments of viral nucleic acids of chainlength larger than 10) in preparative (mg) amounts.

Methods Employed: Various procedures for the preparation and fractionation of large oligonucleotides from RNA (MS2 virus) and DNA (fd virus) were investigated. 1) A procedure for the strip chart counting of ^{32}P-labeled oligonucleotides fractionated by polyacrylamide gel electrophoresis was studied. 2) Methods for growth of MS2 virus with improved yields were investigated (see AM - 15500 LNE). 3) The action of S_1 nuclease from Aspergillus oryzae on single-stranded nucleic acids with partial helical content (see LNE-10, 1973-1974) was examined further using two pure tRNA's as substrate. 4) A major and a minor endonuclease from the same source as S_1 nuclease, first described by Ando, were characterized. 5) Attempts were discontinued to purify two DNAses from strain 2645 (Institute of Applied Microbiology, Osaka, Japan) because of non-reproducible yield and stability of the two enzymes. 6) Mapping procedures for ^{32}P-labeled oligonucleotides as well as for unlabeled compounds were studied.

Major Findings: Strip chart counting of ^{32}P-labeled compounds in gels allows recovery of about 0.7-1% of the cpm detectable by Cerenkov radiation in a scintillation counter. The low yield is due to the lower efficiency of gas flow counting compared to scintillation counting, the geometry of the detector assembly and quenching by the gel itself. However strip chart counting is nondestructive, requires no X-ray films, and takes less time than slicing of gels: 3-4 gel strips per hour may be readily scanned.

Higher yields of MS2 than previously reported were obtained by using enriched media, better aeration and inoculation of E. coli at higher cell densities. In the 300-liter fermentor of the pilot plant yields of 75-80 g of MS2 per run are thus feasible.

Pure arg and f-met-tRNA were both halved by S_1 nuclease (at 4^o and in 0.3 M salt) at a site at or near the middle of the molecule in 30-40% yields. This was determined from molecular weight estimates by electrophoresis through 98% formamide gels at 30^o, using 16s rRNA, 5s and tRNA as standards. The mol. wts. so obtained were 12,500 and 13,500, and 13,000 and 14,000 respectively for f-met- and arg-tRNA. Electrophoresis through 7 M urea at 4^o rather than through formamide gave spurious results, such as the appearance of compounds of lower mobility than tRNA itself. To obtain such tRNA splits, a large excess of S_1 nuclease was required, and once obtained, the halves were quite stable to further S_1 hydrolyses. This agrees with the finding by others that the codon loop (near the middle) protrudes more from the folded tRNA than the other loops. The action of S_1 nuclease on both tRNAs will be further investigated by sequencing of the relevant compounds.

The Asp. oryzae strain that produces S_1 nuclease also contains other DNases, as observed by Ando during the purification of S_1. These two enzymes were isolated as byproducts during the purification of S_1. The major enzyme is an endonuclease, requires Mg^{+2} or Mn^{+2} rather than Zn^{+2}, has a pH optimum near 5, is inhibited by RNA and yields mainly dinucleotides upon exhaustive digestion. The enzyme has not preferential base specificity and hydrolyzes calf thymus DNA at a rapid rate. The minor enzyme has not been characterized. This project is not completed.

Sanger's mapping procedure for ^{32}P-labeled oligonucleotides employs electrophoresis on cellulose acetate strips in the first dimension. The fractionated compounds are then transferred by elution with water to DEAE-paper or DEAE-cellulose thin layer plates. Strip chart counting of cellulose acetate strips after blotting shows that ^{32}P transfer is not quantitative and that the efficiency of tansfer varies for different oligonucleotides. For this reason, other media for the first dimension and/or other media that do not require transfers are under investigation.

Significance to Medical Research and the Program of the Institute: Strip chart counting of ^{32}P gels and improved yields for MS2 production should be of value for studies on nucleic acid structure and function. The ability of S_1 nuclease to halve tRNAs has been observed with other enzymes but with much lower yields. The stability of the halves indicates that S_1 nuclease should be useful for sequencing of nucleic acids with partial helical content.

Proposed Course of Project: Work with S_1 nuclease will be concluded. The proposed course includes studies on the action and purification of restriction endonucleases and DNA ligases.

Honors and Awards: NONE

Publications:

Rushizky, G. W. and Mozejko, J. H.: Strip chart couting of ^{32}P in gel electrophoresis strips. Anal. Biochem. (in press).

Project No. Z01 AM 15105-03 LNE
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Metabolic studies on chromatin: Synthesis, assembly and turnover of chromatin constituents.

Previous Serial Number: NIAMDD-LNE-18

Principal Investigator: Dr. Ronald Seale

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives: The theme of this program is the study of functional aspects of chromatin from a biological standpoint. Of immediate interest are problems related to the control mechanism by which synthesis of DNA and histone proteins is coordinated.

Methods Employed: This work is concerned with the study of events of replication in eukaryotic cells; namely the events and mechanism of DNA and chromatin protein biosynthesis, and assembly of these constituents into daughter interphase chromosomes of HeLa cells. The methods employed have primarily involved the isotopic labeling of either the DNA or protein constituents of chromatin, cellular fractionation to isolate the desired component, subsequent fractionation by sedimentation velocity or equilibrium density gradients, or polyacrylamide gel electrophoresis, and counting the radioactivity of fractions obtained. Digestion of chromatin DNA by nucleases has also been extensively employed to examine modes of association of newly synthesized DNA with protein.

Major Findings: (A) Turnover of non-histone chromatin proteins. HeLa cells prelabeled in DNA with ^{14}C thymidine were labeled with ^{3}H leucine or lysine for long (one generation) and short (30 min) periods, and during the G1 and S phases of the cell cycle of synchronized populations. Following labeling, cells were washed free of isotope and allowed to grow several generations in non-isotopic medium, during which time specific activities of DNA, histone and non-histone chromatin proteins were determined for measure of decay. In all situations, the non-histone decay rate paralleled that of histones and

DNA, i.e., specific activity decline was totally accountable by growth dilution and no turnover was detected.

(B) Replication processes in HeLa cells. Replication of eukaryotes is a manifold process involving not only DNA replication but coordinate cytoplasmic synthesis of chromosomal proteins and assembly of DNA with these proteins. The study of these processes has been facilitated by comparative study of replication in cycloheximide (CH) treated cells, i.e., DNA synthesis in the absence of protein synthesis.

In cycloheximide-blocked cells, DNA synthesis continues at 20% of control rates for 60-90 min and then ceases. By several criteria including the demonstration of discontinuous DNA synthesis, covalent extension of nascent DNA at replication forks and the absence of breakdown of non-replicating DNA, this appeared to be true replicative DNA synthesis rather than repair.

The DNA replicative process is defective, however, because fragments with a 21S modal distribution accumulate and are not converted to high molecular weight DNA. Upon reversal of inhibition, these fragments are eventually incorporated into high molecular weight DNA by a chain elongation mechanism, but this process takes about 3 hours as compared to 40-60 minutes in untreated cells.

Pool chromatin proteins were demonstrated to exist by prelabeling proteins with a brief (5 minute) pulse of labeled amino acid followed by addition of CH after which radioactivity in both histones and nonhistone chromatin proteins increased by as much as 100% during 60 minutes of residual DNA synthesis. Resolution of these proteins on polyacrylamide gels revealed that the low molecular weight histone, f2al, was preferentially incorporated during CH inhibition. This pool of proteins, although not measured, probably represents about 0.2% of total chromatin proteins.

Since protein synthesis in CH is effectively zero, chromatin, normally consisting of a complex of DNA and protein, should be demonstrably deficient in protein. Using deoxyribonuclease digestion as a relative measure of the amount of protein complexed to DNA, this deficiency was verified. DNA synthesized is digested to a greater degree than control chromatin when the digestion was performed either with DNase I or micrococcal nuclease. During recovery from CH inhibition, this protein deficiency is replenished but the process takes 3 hours for completion.

In control cells briefly pulse-labeled with ^{3}H-thymidine, the nascent chromatin DNA is also more nuclease sensitive than mature chromatin DNA. This nuclease sensitivity progressively increases to the level of controls and is complete in about 15 minutes. The maturation time, taken to represent protein (histone) accumulation on nascent DNA and corresponds with the time necessary to complete replicons, or replication units of eukaryotic DNA.

Micrococcal nuclease limit digests of chromatin yield a rather simple pattern in polyacrylamide gels of polynucleotides protected from digeston by associated histones. Comparisonof the controll digest polynucleotide pattern to that of cycloheximide-chromatin and nascent pulse-labeled chromatin all showed that all these types of chromatins had the same spectrum of nuclease-resistant polynucleotides, regardless of the total nuclease sensitivity of the chromatin.

Because cycloheximide-chromatin and nascent chromatin DNA have increased susceptibility to nuclease digestion presumably representing decreased protein content, it was predicted that the buoyant density of these preparations in CsCl should be intermediate between those of free DNA (ρ=1.7) and control chromatin (ρ=1.35). Instead, it developed that in both experimental situations the chromatin banded as a light satellite to control chromatin. In control cells, this light satellite exists only for about one minute after which conversion the density of control chromatin is complete. The reason for the decreased density is probably due to association with macromolecules involved with replication (membrane attachment, replication complex, unwinding protein?) but is currently not known.

In summary, we now have experimental evidence to describe early events in eukaryote replication. DNA is replicated at sites in the DNP complex which leaves pre-existent chromatin proteins in place. DNA synthesis continues until completion of a replicating unit (replicon) of DNA is complete and a new complement of chromatin protein is then added to complete the daughter chromosome at that locus. During S-phase, adjacent loci eventually are all replicated resulting finally in a duplicate of the parental chromosome.

Significance to Bio-Medical Research and the Program of the Institute: Duplication of genetic material is a fundamental process common to all prokaryotes, eukaryotes and viruses, regardless of origin. Replication is an absolute prerequisite for cell division, embryogenesis, tissue differentiation, healing and regeneration, and many types of transformation and oncogenic states. Thus, information brought to bear on eukaryote replication and the control processes which trigger and regulate it is of fundamental value.

Keyword Descriptors: HeLa chromatin; replication; DNA-protein assembly.

Honors and Awards: None

Publications:

Seale, R. L.: Conservation of non-histone chromatin proteins during growth in HeLa cells. Biochem. Biophys. Res. Commun. 63, 140-148 (1975)

Seale, R. L. and Simpson, R. T.: Effects of cylcoheximide on chromatin biosynthesis. J. Mol. Biol. 93 (in press).

Seale, R. L.: Assembly of DNA and protein during replication in HeLa cells. Nature (in press).

Project No. Z01 AM-15200-15 LNE
1. Nutrition and Endocrinology
2. Vitamin Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on folic acid.

Previous Serial Number: NIAMDD-LNE-1

Principal Investigators: Dr. B. T. Kaufman and Dr. Verne Kemerer.

Other Investigators Ms. R. C. Gardiner and Ms. M. K. Romine.

Cooperating Units: None

Man Years:

Total:	4.0
Professional:	2.0
Other:	2.0

Project Description:

Objectives: To isolate and determine the biochemical properties of enzymes concerned with the metabolism of folic acid, especially dihydrofolic reductase from chicken liver, beef liver, yeast, bacteria and certain leukemic tumor cells. To study the interaction of these enzymes with agents known to produce activation, as well as with certain anti-cancer drugs which result in inactivation. To isolate and determine the chemical and biological properties of the naturally occurring forms of folic acid in various cells and tissues.

Methods Employed: Standard chemical and biochemical techniques appropriate for the purification and fractionation of proteins and vitamins are used in these projects in conjunction with various methods of chemical, physical, enzymatic and microbiological analysis.

Major Findings: Beef liver dihydrofolate reducatase has been purified to homogeneity as previously described using a methotrexate affinity column followed by gel filtration to remove several higher molecular weight proteins and hydroxylapatite to remove bound substrates. The purity and molecular parameters of the enzyme have been investigated.

The enzyme is homogenous by a variety of methods. The disc gel electrophoretic pattern consists of a single band. Occasionally a faint contaminating band can also be observed. This band appears to correspond to a small enzymatically active peak observed in the electrofocusing profile with an isoelectric point of 5.7. This minor band probably results either from the deamidation of a glutamine residue or from the binding of trace amounts of substrate. Either case would increase the negative charge on the protein. The Scliern sedimentation pattern shows a single symmetrical band. From the equilibrium sedimentation data, a plot of ln C vs R^2 in linear which is also indicative of a single species. Methotrexate titrations show an inhibitor - protein stoichiometry approaching 1.

The isoelectric point of beef liver dihydrofolate reductase is 6.8. This is quite different from those previously observed for other animal reductases, i.e., chicken liver, 8.4, and the fR8 lymphoma 9.4.

The cancer drug, Methotrexate, exhibits the typical titration pattern characteristic of a stoichiometric interaction. The degree of inhibition is linear up to approximately 95% of the original activity. This titration shows that the binding of one mole of methotrexate is completely inhibitory. The slope of the titration curve yields a turnover number of 578 moles of dihydrofolate reduced per min per mole of enzyme.

Notable features of the amino acid composition of beef liver dihydrofolic reductase are a single cysteine and low amounts of histidine, tryptophan, methionine, tyrosine and alanine. A minimal molecular weight of 22,530 was calculated from the cysteine content. A large preponderance of acidic residues exists relative to the basic amino acids. The tyrosine, tryptophan and methionine content of the beef reductase are quite similar to those for chicken liver, L1210 lymphoma and *E. coli* enzymes. Beef liver enzyme is almost identical in amino acid composition to the L1210 reductase.

Sedimentation studies reveal that at very low protein concentrations, the $S_{20,w}$ is 2.08 but the $S^{o}_{20,w}$ extrapolated from higher protein concentrations is 2.39. It thus appears that the enzyme is undergoing some transitions at lower concentrations which make it less symmetrical. The molecular weight calculated from meniscus depletion experiments ± 400. The linearity of the data indicate the presence of only one molecular species and that the system is probably not concentration dependent nor behaving in a non-ideal fashion.

The molecualr weight determined by SDS gel electrophoresis of 21,300 indicates that the enzyme is composed of a single subunit. The diffusion coefficient was measured directly and was also calculated from the effective molecular weight obtained by equilibrium sedimentation and the extrapolated $S^{o}_{20,w}$. The average diffusion coeffieient of 10.93 cm^2-sec^{-1} and an average frictional coefficient of 1.043 were obtained by combining these two independently determined diffusion coefficients. From this frictional coefficient a maximal axial ratio of 2 is possible.

If a spherical shape is assumed, a maximal hydration of 0.1 g-g^{-1} is permitted. The radius of this hydrated sphere is 19.2 Å. Since the amount of hydration is quite low, it is probable that the enzyme has an axial ratio very close to unity.

The molar extinction coefficient for the beef liver dihydrofolate reductase was determined refractometrically to be 27,280. An extinction coefficient calculated from the amino acid composition is 14% lower. Since the extinction coefficients of tyrosine and tryptophan increases in less polar solvents, it is probable that the aromatic residues in this enzyme are buried in hydrophobic regions of the moelcule resulting in the higher extinction coefficient.

Significance to Bio-Medical Research and the Program of the Institute: Dihydrofolic reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Thus, the understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and in cancer chemotherapy. Folic acid, as such, most probably does not occur in nature. Thus, the knowledge of chemical structure and biological properties of the more complex natural form will result in a better understanding of the biochemistry of this vitamin.

Proposed Course of Project: Now that for the first time reasonably large amounts of completely pure enzyme from both beef and chicken are available, investigations can begin to examine the physical properties and mechanism of these two liver dihydrofolic reductases with particular emphasis on the nature of the activations and inhibitions.

Keyword Descriptors: Purification and properties of dihydrofolic reductase.

Honors and Awards: NONE

Publications:

Kaufman, B. T.: Methotrexate-agarose in the purification of dihydrofolate reducatase (1975) Methods in Enzymol. 34, 272-281.

Kaufman, B. T. and Kemerer, V. F.: Purification and characterization of beef liver dihydrofolate reductase. (in press).

Project No. Z01 AM15300 -10 LNE
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Studies Related to Nutrition

Previous Serial Number: NIAMDD-LNE-8

Principal Investigator: Dr. J. G. Bieri

Other Investigators: Dr. R. E. Poukka Evarts

Cooperating Units: None

Man Years:

Total:	5
Professional:	2
Others:	3

Project Description:

Objectives - To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed - Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

Major Findings - Metabolism and function of fat-soluble vitamins. Studies have continued on the metabolism of α-tocopherol and the assessment of human vitamin E status. α-Tocopherol exchanges rapidly between red cells and plasma, and the distribution at equilibrium has been found to be affected by a number of factors. Use of heparin as an anticoagulant increased the red cell concentration 2-3 fold compared with citrate or oxalate as anticoagulants. Substituting bovine serum albumin for plasma increased the red cell uptake about 50 times. Of physiological interest was the observation that the plasma lipid content affects the distribution of α-tocopherol between red cells and plasma. For these studies, hyperlipemic plasma was obtained from genetically obese rats and hypolipemic plasma was taken from rats fed orotic acid. Incubation of these plasmas with cells from normal rats showed that at equilibrium the ratio of α-tocopherol concentration in cells:plasma was inversely related to the plasma lipid content.

In view of these findings, studies were conducted to determine the effect of plasma lipid level on the deposition of α-tocopherol in tissues. Genetically obese rats and rats fed orotic acid were given equal doses of α-tocopherol daily for several weeks. When sacrificed, obese rats had plasma total lipids and α-tocopherol levels three times those of control, non-obese rats. Tissue concentrations of α-tocopherol, however, were significantly lower in obese rats. Due to their large adipose mass, obese rats had about 6 times more total body α-tocopherol than did non-obese controls. Rats fed orotic acid had plasma total lipids and α-tocopherol levels one-third those of control rats; tissue concentrations of α-tocopherol were significantly lower in rats fed orotic acid.

Significance to Biomedical Research - These studies indicate that the evaluation of body vitamin E status is more complex than generally believed, and that two important factors are the level of plasma total lipids and the extent of body adiposity.

Proposed Course - Studies will continue on the metabolism and cellular function of vitamin E and of related nutritional factors.

Keyword Descriptors - vitamin E nutritional status
vitamin E and plasma lipids
α-tocopherol storage
obese rats
hyperlipemia and vitamin E

Honors and Awards - President, American Institute of Nutrition 1974-75
Distinguished Alumni Fellow, Pennsylvania State University, 1975

Publications:

Bieri, J. G. & Evarts, R. Poukka: Vitamin E activity of γ-tocopherol in the rat, chick and hamster. J. Nutr. 104: 850-857, 1974.

Bieri, J. G.: Recommended Dietary Allowances--Vitamin A; vitamin E: Recommended Dietary Allowances, Eighth Edition. Washington, D. C., National Academy of Sciences, 1974, pp. 49-54, 56-61.

Bieri, J. G. & Evarts, R. P.: Gamma Tocopherol: Metabolism, biological activity and significance in human vitamin E nutrition. J. Am. Clin. Nutr. 27: 980-986, 1974.

Evarts, R. P. & Bieri, J. G.: Ratios of Polyunsaturated Fatty Acids to α-Tocopherol in Tissues of Rats Fed Corn or Soybean Oils. Lipids 9: 860-864, 1974.

Bieri, J. G. & Evarts, R. P.: Vitamin E adequacy of vegetable oils. J. Am. Diet. Asso. 66: 134-139, 1975.

Bieri, J. G. & Evarts, R. P.: Effect of Plasma Lipid Levels and Obesity on Tissue Stores of α-Tocopherol. Proc. Soc. Exp. Biol. & Med.: in press.

Project No. Z01 AM15301 - 10 LNE
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Studies of Cytochromes and Sub-Cellular Particles

Previous Serial Number: Included in NIAMDD-LNE-8

Principal Investigator: Dr. J. N. Williams, Jr.

Other Investigators: Mrs. S. L. Thorp

Cooperating Units: None

Man Years:

Total	2
Professional:	1
Other:	1

Project Description:

Objectives: To obtain a better understanding of energy metabolism by elucidating the subcellular biochemical reactions which generate and utilize energy.

Methods Employed: Liver is separated into its cellular components by differential centrifugation and the sub-cellular particles analyzed by various chemical and physical measurements for cytochromes and enzymes. Animals are treated by dietary manipulation and also by administration of drugs and metabolites.

Major Findings: Since glucagon stimulates the metabolic activity of liver mitochondria as well as other sub-cellular particles studies were performed to assess the effect of this hormone on mitochondrial cytochromes and protein. Rats were injected with glucagon for 2 days and the liver mitochondria subsequently analyzed spectrophotometrically for protein and cytochromes aa_3, b, c_1, and c, and also cytochrome oxidase. Whether expressed on the basis of liver or body weight, all cytochromes were decreased by glucagon treatment, the magnitude of the decreases generally paralleling the diminution in protein.

Significance to Biomedical Research: These studies provide further evidence as to how hormones can profoundly affect energy metabolism, and indicate another metabolic effect of glucagon.

Proposed Course: Project discontinued in 1974 due to retirement of the principal investigator.

Publications:

Williams, J. N., Jr. and Thorp, S. L.: Biochemical Studies on Sub-fractions of Rat Liver Mitochondria. Life Sci. 15: 695-709, 1974.

Williams, J. N., Jr. and Thorp, S. L.: Effects of Glucagon upon Rat Liver Mitochondria in Vivo. Proc. Soc. Exp. Biol. & Med. 147: 803-806, 1974.

Project No. Z01 AM 15302 -05 LNE
1. Nutrition and Endocrinology
2. Nutritional Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Studies of Hepatic and Intestinal Function

Previous Serial Number: Included in NIAMDD-LNE-8

Principal Investigator: Dr. H. G. Windmueller

Other Investigators: Mr. A. E. Spaeth

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: To increase our understanding of the biochemical transformations occurring in the liver and small intestine. To define the nutritional requirements of these organs, to study the effects of hormones and nutritional variables, and to study the physiological relationship of these organs to other tissues in the body.

Methods employed: Surgical and biochemical techniques are used. Biochemical determinations are made on isolated rat livers and small intestine perfused with blood under physiological conditions. Also used are rats surgically prepared with indwelling lymph or bile cannulae as well as a supradiaphragmatic rat preparation. The metabolic and transport activity of the organs is assessed by the use of radioactive isotopes and measurements of tissue and blood enzyme and metabolite concentrations.

Major findings: In previous studies with an isolated preparation of rat intestine perfused with blood we showed that mucosal cells of the small intestine extract large amounts of glutamine, but no other amino acid, from the circulation. Rapid metabolism of the glutamine leads to recovery of the carbon in venous blood as CO_2(55%), proline (5%), citrulline (6%), and organic acids, mostly lactate (18%), and recovery of the nitrogen in ammonia (23%), alanine (33%), citrulline (34%) and proline (10%). One-third of the respired CO_2 produced by intestine in fasted animals comes from metabolism of arterial glutamine. Utilization of arterial glutamine was demonstrated in a variety of animal species.

We have now developed a simpler *in vivo* preparation for studying the fate of glutamine and glutamate when transported from the intestinal lumen. A 3-5 cm segment of rat jejunum *in situ* with intact blood supply is isolated with ligatures, the single vein draining the segment is cannulated, and all the blood is collected for analysis. Normal arterial pressure in the animal is maintained by a continuous infusion of replacement blood from donor rats. L-[U-^{14}C]glutamine introduced at a physiological concentration into the lumen of such a preparation was completely absorbed in 60 min. but only 35% appeared unchanged in the venous blood. The remaining ^{14}C was recovered in the same products and in very nearly the same proportions as observed with the isolated, blood perfused preparation. L-[U-^{14}C]glutamate disappeared from the lumen more slowly (90% in 100 min) and only 2% was transported unchanged, the remainder giving rise to the same products as glutamine, with the exception that no ammonia was produced. We conclude that: (a) metabolism of glutamine entering mucosal cells from the blood or the lumen follows a similar pathway and proceeds *via* glutamate; and (b) all ingested glutamate and most glutamine, which together comprise 15-30% of the amino acid residues of dietary proteins, are metabolized to CO_2, organic acids, and other amino acids during intestinal transport.

We also measured the individual and combined rates at which intestine metabolizes glutamine from the lumen and from arterial blood, since gut is unique in its potential for deriving nutrients from both sources. Metabolism of arterial glutamine averaged 200 nmoles/min/g tissue in fasted animals and fell to 140 nmoles/min/g tissue when glutamine was perfused through the lumen. Metabolism of the luminal glutamine averaged 180 nmoles/min/g tissue, with the combined rates, 320 nmoles/min/g, being 60% greater than the metabolic rate of arterial glutamine alone.

Significance to Biomedical Research and the Program of the Institute: These studies contribute basic information on the metabolism of the intestine and on the digestive process. The metabolism of dietary glutamine and glutamate and circulating glutamine produced by other tissues provides an important source of energy for the small intestine, results in the addition of ammonia, alanine, ornithine, citrulline, and proline into the portal blood and may serve a special yet undefined biochemical role in this tissue.

Proposed Course: This unique intestinal preparation will be used to identify other nutrients, both dietary and from the circulation, that undergo metabolism in the intestine.

Keyword Descriptors: Glutamine
Glutamate
Amino acid metabolism
Intestinal metabolism
Intestinal transport

Publications:

Windmueller, H. G. and Spaeth, A. E.: Uptake and Metabolism of Glutamine by the Small Intestine. J. Biol. Chem. 249: 5070-5079, 1974.

Herbert, P. N., Windmueller, H. G., Bersot, T. P. and Shulman, R. S.: Characterization of Rat Apolipoproteins. I. The Low Molecular Weight Proteins of Rat Plasma High Density Lipoproteins. J. Biol. Chem. 249, 5718-5724, 1974.

Project No. Z01 AM 15400-01 LNE
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hormones, lipoprotein lipase and fat metabolism

Previous Serial Number: NIAMDD-LNE-5

Principal Investigators: Drs. Robert O. Scow and Sidney S. Chernick

Other Investigators: Dr. Peter M. Spooner, Staff Fellow
Dr. Angelo A. Ucci, Jr., Research Associate

Cooperating Units: Z01 AM 15401

PL-480 Agreement No. 02-009-1 with Department of Physiology
University of Skopje
Skopje, Yugoslavia

Man Years:

Total:	6
Professional:	2
Other:	4

Project Description:

Objectives: To determine influence of hormones and other factors on the metabolism of fat in liver, adipose tissue, lactating mammary gland, crop sac and other tissues of different animals. To determine how hormones regulate lipoprotein lipase activity in tissues.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with hormones or other substances. The effects of the above on lipid metabolism and lipoprotein lipase activity in different tissues are determined with in vivo and in vitro studies using conventional and radioisotopic techniques. Effects of hormones added in vitro are also studied.

Major Findings: Mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland: Dietary fatty acids are used by lactating mammary gland for synthesis of milk triglyceride. They are transported in blood as triglyceride in chylomicrons and their uptake by mammary gland is regulated by lipoprotein lipase. Lipoprotein lipase activity is increased in mammary gland and decreased in adipose tissue during lactation, thus diverting dietary fatty acids from body fat stores to the mammary gland for the synthesis of milk triglyceride. Studies reported earlier showed in lactating rats that these changes in lipoprotein lipase activity are mediated through prolactin secretion by the anterior pituitary gland. Studies by others, in pseudopregnant rabbits, showed that the effect of prolactin on lipoprotein lipase in mammary gland requires the continued production of a short-lived messenger RNA.

The present study was initiated to determine the mechanism of hormonal regulation of lipoprotein lipase activity in mammary gland using a mammary tissue preparation, such as explants, capable of responding *in vitro* to prolactin. During the past 6 months, studies have been made in both intact and hypophysectomized immature rats to determine the pattern of hormonal priming (with growth hormone, estrogen, progesterone and glucocorticoids) necessary for a rapid effect of prolactin on lipoprotein lipase in the gland. The findings to date indicate that growth and development of the mammary gland occur in immature animals without any change in lipoprotein lipase activity in the tissue. The possibility that prolactin and other hormones may also be necessary for priming the gland is being investigated.

Effect of prolactin on lipoprotein lipase activity in crop sac and adipose tissue of pigeons: Crop "milk" is an oily, cheese-like substance, consisting of desquamated epithelial cells, produced in the crop sac of pigeons and doves for feeding their young. Since crop "milk" has a high fat content, 12%, and its formation is dependent on prolactin, the possible role of lipoprotein lipase in this process was investigated in adult pigeons. It was found that prolactin injections for 4 days increased lipoprotein lipase activity 9-fold in the crop sac and less than 1-fold in adipose tissue. Prolactin injections also stimulated growth of the crop sac and production of crop "milk". The finding of increased lipoprotein lipase activity in crop sac of prolactin-treated pigeons suggests that blood triglyceride may be used by crop sac for the formation of crop "milk" lipid.

Effect of prolactin on blood lipids in pigeons: Since the diet of pigeons is usually low in fat and high in carbohydrate, it is likely that some of the fatty acids in blood triglyceride are derived from synthesis *de novo*. Studies elsewhere showed that fatty acid synthesis in normal and prolactin-treated pigeons occurs primarily in the liver and that fatty acids stored in adipose tissue are derived mostly from triglyceride in blood. It was also found that fatty acid synthesis in liver, the concentration of fatty acids in blood, and accumulation of fat in adipose tissue are increased by prolactin. The proportion of fatty acids in blood derived from diet and that from synthesis in liver, as well as the class of plasma lipoprotein involved in their transport, were not studied. The present investigation was initiated to study these aspects of lipid transport, as well as the source of fatty acids secreted in crop "milk" lipid and the role of lipoprotein lipase in their uptake by crop sac.

It was found that daily injections of prolactin increased the serum triglyceride concentration of pigeons 2-fold in 2 days and 3-fold in 7 days, and doubled the phospholipid and cholesterol concentrations within 7 days. Ultracentrifugation studies showed that most of the increase in triglyceride content of serum occurred in the d<1.006 fraction. The startling finding concerning this fraction was that it contained nearly equimolar concentrations of triglyceride, phospholipid and cholesterol, suggesting that it may be composed mostly of remnants of very low density lipoproteins (or chylomicrons). Electron microscopic studies showed that the particles in this fraction ranged in diameter from 125 to 1800 Å, and that some resembled in appearance remnants of chylomicrons produced, in vivo and in vitro, in this laboratory.

Significance to Biomedical Research and the Program of the Institute: There is considerable evidence now that lipoprotein lipase is involved in the removal of triglyceride from blood and that the enzyme acts in the capillaries of extrahepatic tissues. Recent studies in this laboratory have shown that prolactin greatly increases the activity of this enzyme in rat mammary gland and pigeon crop sac and lowers the activity in rat adipose tissue, and that these changes in enzyme activity directly affect the uptake of triglyceride fatty acids by these tissues. It was also found that uptake of plasma cholesterol by perfused lactating mammary gland is, likewise, dependent on the activity of lipoprotein lipase. This is the first demonstration that this enzyme is involved in uptake of plasma cholesterol by extrahepatic tissue.

Our recent studies also show that prolactin injections cause in pigeons a large increase of all lipids in the VLDL fraction of plasma and electron microscopic studies indicate that the particles in this fraction resemble chylomicron "remnants" produced in this laboratory. The possible relationship of these findings in prolactin-treated pigeons to that seen in patients with LCAT (lecithin-cholesterol-acyl-transferase) deficiency awaits further study.

Proposed course: The mechanism of action of prolactin in the regulation of lipoprotein lipase activity will be studied in both rodent mammary tissue and pigeon crop sac, using explant techniques. The role of plasma lipids and lipoprotein lipase in the formation of pigeon crop "milk" will also be studied. The origin of the "remnants" found in the VLDL fraction of plasma of prolactin-treated pigeons will be studied in detail and related to clinical conditions in which such particles appear.

Keyword Descriptors:

Hormones and fat metabolism
Hormones and lipoprotein lipase
Prolactin and lipoprotein lipase
Lipoprotein lipase and cholesterol uptake
Lactating mammary gland
Crop sac

Plasma VLDL
Plasma cholesterol
Plasma triglyceride
Chylomicrons

Honors and Awards:

Dr. Robert O. Scow presented the George Lyman Duff Memorial Lecture at the 1974 Annual Meeting of the Council on Arteriosclerosis of the American Heart Association.

Publications:

Bates, R.W.: Bioassay Methods for Prolactin in Blood. In Josimovich, J.J. Jr. (Ed.): Lactogenic Hormones, Fetal Nutrition and Lactation. New York, John Wiley and Sons, 1974, pp. 85-95.

Bates, R.W. and Garrison, M.M.: Hormonal interactions among GH, ACTH, cortisol and dexamethasone upon size of kidney, liver and adrenal. Proc. Soc. Exp. Biol. Med. 146: 725-731, 1974.

Bates, R.W. and Garrison, M.M.: Daily changes in concentration of pancreatic and serum insulin and of blood glucose during 5 days of treatment of rats with growth hormone, ACTH, cortisol, dexamethasone, and tolbutamide alone and in combination. Metabolism 23: 947-965, 1974.

Garrison, M.M. and Scow, R.O.: Effect of prolactin on lipoprotein lipase in crop sac and adipose tissue of pigeons. Am. J. Physiol. 228:   1975.

Spirovski, M.Z., Kovacev, V.P., Spasoska, M. and Chernick, S.S.: Effect of ACTH on lipolysis in adipose tissue of normal and adrenalectomized rats in vivo. Am. J. Physiol. 228: 382-385, 1975.

Project No. Z01 AM 15401-03 LNE
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Transport of lipid, hormones and enzymes through cells and membranes

Previous Serial Number: NIAMDD-LNE-6

Principal Investigators: Drs. E. Joan Blanchette-Mackie and Robert O. Scow

Other Investigators: None

Cooperating Unit: Z01 AM 15402

Man Years:

Total:	3
Professional:	1
Other:	2

Project Description:

Objectives: To study the structure and metabolism of cells that synthesize and secrete plasma lipids and hormones. To study the transport of lipids, hormones and enzymes across the capillary endothelium, basement membrane and extracellular space in different kinds of tissues. To study the uptake of lipids, hormones and enzymes by different kinds of cells. To study the structure of cellular membranes and their role in lipid metabolism. To correlate structural and biochemical changes induced in cells and tissues by various means.

Methods Employed: Animals of different ages and in various physiological states (fasting, pregnant, lactating, diabetic) are used to study with biochemical and morphological techniques the synthesis and secretion of lipoprotein triglyceride by liver and intestines, and the utilization of plasma triglyceride by adipose tissue, mammary gland and muscle. Perfused adipose and mammary tissues are used to localize with cytochemical techniques the sites of action of lipoprotein lipase and the mode of transport of fatty acids between blood and tissue cells. Perfused tissues are also used to determine with immuno-cytochemical techniques the distribution and site of synthesis of lipoprotein lipase and the transport of hormones across capillary endothelium and extracellular space. Fatty livers of pancreatectomized animals are used to study uptake of fatty acids and accumulation of triglyceride within cellular membranes. Isolated chylomicrons and purified lipoprotein lipase incubated in vitro are used to study the morphological changes in chylomicrons induced when the triglyceride core is reduced by lipolysis.

Major Findings: Effects of lipoprotein lipase on chylomicrons in vitro. Earlier studies in this laboratory showed that purified lipoprotein lipase, obtained from bovine milk, readily hydrolyzed triglyceride of chylomicrons to FFA and glycerol if sufficient albumin was present in the medium to bind all of the FFA produced. However, when albumin was limited, it was found that lipolysis was markedly reduced and that FFA and monoglyceride, products of lipolysis, accumulated in the chylomicrons. Structural analysis with transmission electron microscopy (of whole mounts, sections and freeze fracture replices) and scanning electron microscopy showed that these chylomicrons also contained aqueous spaces lined with a lipid monolayer. Sometimes, the aqueous spaces contained whorls of bilayered lamellae which appeared to be continuous with both the surface film of the chylomicron and the lipid monolayer lining the aqueous space. These findings suggest that the monolayer lining the spaces may be an extension of the surface film, although different in composition, formed by the accumulation of FFA and monoglyceride at the triglyceride-water interface, and that this monolayer can extend itself by forming a spiralling fold within the aqueous space. Thus, hydrolysis of triglyceride forms amphiphilic acyl lipids, FFA and monoglyceride, which can locate and move within the interface between lipid and water. It is proposed that this translocation of lipid is probably the principal effect of the action of lipoprotein lipase on chylomicrons and that it is essential for the transfer of fatty acids from chylomicrons across capillary endothelium.

Secretion of milk lipid by mammary gland. Morphological observations in mammary gland of lactating rats indicate that the Golgi apparatus plays a major role in the release of milk lipid from mammary alveolar cells. Lipid droplets in the cells become surrounded by Golgi vesicles at the apex of the cells. The membranes of the vesicles then fuse with each other and with the apical plasma membrane of the cells resulting in the secretion of lipid droplets. The Golgi vesicles are also the origin of membranes surrounding milk lipid droplets in the alveolar lumen as well as part of the apical plasma membrane of the alveolar cells.

Fatty liver in diabetic rats. Fatty liver and hypertriglyceridemia as well as severe diabetes, develop within 24 hours following "total" pancreatectomy in fasting rats. Accumulation of triglyceride in the liver results from increased mobilization of fatty acids from adipose tissue, and the high triglyceride concentration in blood, up to 6 mM, reflects increased lipoprotein secretion by the liver. Electron microscopic study of livers from these animals showed that the lumina of endoplasmic reticulum in the parenchymal cells contained small lipid particles (400 Å) which had structural continuity with membranes of the endoplasmic reticulum. Also, the large lipid droplets within the cells contained, in the triglyceride core, whorled bilayered lamellae which were similar to those found in the aqueous spaces of partially lipolyzed chylomicrons (see above). The lamellae in the lipid droplets also appeared to be continuous with the membranes of the smooth endoplasmic reticulum. These findings suggest that amphiphilic lipids such as fatty acids locate within the lamellae of the membranes of the endoplasmic reticulum, whereas the triglyceride formed accumulates in the hydrophobic zones of the membranes, between the lamellae.

Significance to Biomedical Research and the Program of the Institute. Alterations in the metabolism of plasma triglyceride occur in various hyperlipemic states. In some, synthesis and secretion of lipoproteins are abnormal, whereas in others, uptake is reduced, sometimes with changes in lipoprotein lipase activity. Localization of lipoprotein lipase synthesis and activity in tissues and study of the interaction of lipoprotein lipase with chylomicrons and other lipoproteins of plasma will provide valuable information concerning factors that regulate removal of triglyceride from the blood stream.

Proposed course. We plan to use the freeze etching technique, combined with electron microscopy, to study the role of amphiphilic lipids in intracellular membrane structure, the transport of lipid from blood to tissue cells, the secretion of lipid by cells and the interaction of lipoprotein lipase with chylomicrons. The freeze etching technique is especially suitable for these studies because it provides surface views of the interphase between layers of lipid molecules, as in cell membranes and between the surface film and core of chylomicrons. In addition, the technique preserves lipid because it does not require chemical dehydration of specimens.

Immuno-cytochemical techniques will be developed for localizing lipoprotein lipase. They will be used to study distribution of the enzyme in mammary and adipose tissue during various physiological states (e.g. pregnancy, lactation, fasting), to localize the enzyme in capillary endothelium, and to study the interaction of the enzyme with chylomicrons.

Keyword Descriptors:

Membranes
Lipid bilayers
Amphiphilic lipids
Triglyceride
Chylomicrons
Lipoprotein lipase
Capillaries
Liver
Mammary gland
Adipose tissue
Lipid transport
Electron microscopy
Autoradiography
Freeze-fracture

Honors and awards: None

Publications:

Scow, R.O., Blanchette-Mackie, E.J., Mendelson, C.R., Hamosh, M. and Zinder, O.: Incorporation of Dietary Fatty Acids into Milk Triglyceride: Mechanism and Regulation. In Kretchmer, N., Rossi, E. and Sereni, F. (Eds.): Modern Problems in Paediatrics, Vol. 15. Basil, Karger, 1975, pp. 31-45.

Project No. Z01 AM 15402-01 LNE
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pharyngeal lipase and digestion of dietary fat

Previous Serial Number: NIAMDD-LNE-5

Principal Investigator: Dr. Robert O. Scow

Other Investigator: Dr. Sidney S. Chernick

Cooperating Unit: Ministry of Education, Finland
Department of Pediatrics
University of Oulu
Oulu, Finland

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: To purify and characterize pharyngeal lipase isolated from tissues of rats, calves and other species. To study the regulation and mode of secretion of the enzyme by pharyngeal tissues. To determine the role of pharyngeal lipase in the digestion of dietary fat in man and other species.

Methods Employed: Pharyngeal tissues obtained from rats, calves, and other species will be used as source of pharyngeal lipase. Techniques used elsewhere for the isolation and purification of pancreatic lipase will be modified as necessary for extracting the enzyme from pharyngeal tissue. Collaborative studies will be made at the Department of Pediatrics, University of Oulu, Oulu, Finland to determine the role and regulation of pharyngeal lipase activity in neonatal and premature infants.

Major Findings: Last year it was reported that a lipolytic activity was found in esophageal and gastric aspirates from normal adult subjects which was capable of hydrolyzing triglyceride to partial glycerides and FFA at pH 5.5. The lipolytic activity was not present in secretions obtained from the major salivary glands, thus excluding those tissues as a source of the activity found in the esophageal aspirates. It was proposed that in man glands in or near the pharynx secrete a lipase that acts in the stomach to hydrolyze long chain triglycerides to partial glycerides and FFA. Subsequent studies have shown that the lipolytic products formed, especially monoglyceride and FFA, facilitate the emulsification of triglyceride in the stomach.

Collaborative studies recently initiated with Drs. Hans Åkerblom and Kauko Kouvalainen at the University of Oulu, Finland indicate that pharyngeal lipase is present in neonatal and premature infants. Studies are now in progress to determine the role and site of origin of the enzyme.

Significance to Biomedical Research and the Program of the Institute: It was previously thought that ingested triglyceride is emulsified in the stomach by the churning, kneading and squirting movements of the stomach wall, and that enzymic digestion does not occur until fat reaches the duodenum. The above findings, however, indicate that enzymic digestion of fat begins in the stomach, catalyzed by pharyngeal lipase, and that the products formed facilitate emulsification of triglyceride before it leaves the stomach. The importance of this enzyme reaction in patients with abnormal lipid digestion is not yet known.

Proposed course: Studies will be continued to determine the role of pharyngeal lipase in fat digestion and to determine what factors regulate its synthesis and secretion. Studies will also be made to determine the source of the lipase in man. Isolation and purification of lipase from pharyngeal tissues of rats and other species will be initiated.

Keyword Descriptors:

Pharyngeal lipase
Digestion of dietary fat
Triglyceride lipase
Gastric lipolysis
Fat digestion in infants and adults

Honors and awards: None

Publications:

Hamosh, M., Klaeveman, H.L., Wolf, R.O. and Scow, R.O.: Pharyngeal lipase and digestion of dietary triglyceride in man. J. Clin. Invest. 55: 908-913, 1975.

Project No. Z01 AM15500-15 LNE
1. Nutrition and Endocrinology
2. Office of Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Large-scale processing of biological materials.

Previous Serial Number: NIAMDD-LNE-2

Principal Investigator: Mr. D. L. Rogerson, Jr.

Other Investigator: Dr. H. A. Sober

Cooperating Units: None

Man Years:

Total:	4.0
Professional:	1.5
Other:	2.5

Project Description:

Objectives: To provide facilities and services for the processing and/or production of large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials, so that they may be studied under standard laboratory conditions.

Methods Employed: To satisfy the large-scale production needs of NIH investigators, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Two 10-liter, one 50-liter, two 300-liter and one 1100-liter fermentors are utilized for growing microorganisms under a variety of conditions. Bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P and AS-26P centrifuges. Protozoa are concentrated in continuous flow centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gaulin homogenizer. In some instances, a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste using conventional fractionation procedures and the newer chromatographic techniques.

Major Findings: During the past year, despite several shutdowns to the new facilities in Building 6 for corrections, changes and additions, a total of 264 requests were processed for investigators: NIAMDD 132; NICHD 51: NCI 23; BB 22; NHLI 8; NIMH 7; NIAID 4; NINCDS and NIDR 3 each; DRS 2; and others 9. 126.3 Kg quantities (29,990 liters of cultures) of 18 organisms were produced. The microorganisms grown during this period include wild type and mutant strains of *Aerobacter aerogenes, Bacillus amyloliquefaciens, Bacillus pumilus, Bacillus subtilis, Bordetella pertussis, Citrobacter sp., Erwian herbicola, Escherichia coli, Haemophilus aphrophilus, Haemophilus haemolyticus, Haemophilus influenzae, Hameophilus parainfluenzae, HeLa* cells, *Klebsiella cloaceae, Pneumococcus sp., Pseudomonas putida, Pseudomonas sp., Salmonella typhimurium.* Some of the organisms were used as hosts for the production of bacteriophage fd, MS2, φ 80, lambda and T4, others were used to produce extracellular enzymes. Microorganisms were grown 94 times in the two 10-liter, 55 times in the 50-liter; 51 times in the 300-liter and 10 times in the 1100-liter fermentors. Of 210 fermenations, 24 (11%) were contaminated with 19 of these traced to the inocula supplied by the requesting investigators.

Studies, formerly conducted with the NICHD, were continued with the Division of Bacterial Products, Bureau of Biologics, for polysaccharide production by *H. influenzae,* isolation of the enterotoxin proteins from special strains of *E. coli,* and preparation of the protective antigen of typhoid fever from a non-pathogenic organism, *Citrobacter.* Spheroplasts were produced for the NHLI from *E. coli* using 10-, 50-, and 300-liter fermentors in series. The Gaulin laboratory homogenizer was used 35 times to rupture cell suspensions of yeast or bacterial cells.

Large scale processing activities consisted of polynucleotide phosphorylase from 1 kg (dry) *Micrococcus lysodeikticus* cells and ^{32}P labeled *Vitellogenin* from chicken serum for NCI, histamine methylating enzyme from 1.75 kg guinea pig brains for NHLI, isolation of the enzyme Barnase and its inhibitor Barstar from 1100 liter cultures of *B. amyloliquefaciens* and eight two-gallon preparations of hydroxylapatite, a product preferred by some investigators over the commercial substance for its superior resolving power and relatively fast flow rate. Various quantities of plant and animal tissue such as 3.3 kg bovine pituitary glands were either dried, ground and/or extracted in the large ovens, mills and related equipment and solutions such as 50 l of Killam rat virus and 25 l *H. influenzae* b were centrifuged. A 4 l Shikimic acid solution was freeze dried in the Stokes hot pump lyophilizer and other solutions were concentrated including a 300 l culture supernate to 8 liters in the Pfaudler reactor, and two 280 liter solutions in the new hollow fiber Amicon concentrator each to 10 liters.

Keyword Descriptors: Large scale microorganism fermentations and Biological materials processing

Honors and Awards: Group cash award for superior performance during adverse physical working conditions created by moving of Pilot Plant over a period of 18 months.

Publications:

Rogerson, D. L., and Rushizky, G. W.: An improved method for the preparation of MS-2 bacteriophage in large quantities. Anal. Biochem. (in press).

Project No. Z01 AM 15600-20 LNE
1. Nutrition and Endocrinology
2. Office of Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies in experimental nutrition.

Previous Serial Number: NIAMDD-LNE-3

Principal Investigator: Mr. E. G. McDaniel

Other Investigators:
Dr. F. S. Doft, Guest Worker, LNE-NIAMDD
Dr. G. Laqueur, LEP-NIAMDD
Dr. J. C. Smith, VA Hospital, Trace Element Lab.

Cooperating Units: Trace Element Laboratory, VA Hospital

Man Years:

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Major Findings: With Dr. Laqueur we have continued the study of the actions of cycasin and related compounds with respect to production of tumors in rats, and the possible influence of intestinal bacteria upon these actions. It was previously shown that cycasin was non-toxic in adult germfree rats, although the compound routinely produced tumors in conventional rats, in germfree rats infected with certain types of bacteria and in infant germfree rats. The very young rat appears to have the capability of converting cycasin to the toxic form without the presence of bacteria, but this capability is lost very early in the life of the rat. It was also shown that the aglycone of cycasin (MAM), a compound produced from cycasin by the bacterial flora, produced tumors in germfree as well as in conventional rats. Experiments are now in progress to determine whether certain precursors in the *in vitro* synthesis of the aglycone of cycasin are effective in producing tumors in germfree rats as has been shown to be the case with conventional animals. Two of the compounds at present under study dimethyl hydrazine (DMH) and azoxymethane (AOM) have both shown acute toxic effects with respect to liver damage, and the possibility is suggested that this acute toxic effect may be even more marked in germfree than in conventional rats. The compounds are administered by various routes to germfree and to exgermfree or conventional animals, and the metabolites produced will be measured. Animals will then be observed for incidence and types of tumors produced. It is intended to determine at which steps in the enzymatic conversion of cycasin to its aglycone the metabolites become carginogenic and if possible the types of bacteria responsible for such conversions. There is suggestive evidence that intermediate metabolites before the aglycone may be toxic in that the sites of some tumors appear to be different than when the aglycone is administered. It is hoped to determine which steps in the conversion of cycasin to its aglycone may or may not require the presence of bacterial enzymes.

With Dr. Smith experiments have been continued to study the interrelationships of vitamin A and the trace element zinc, and to attempt to determine the way in which zinc seems to control the mobilization and the transport or utilization of vitamin A. In a state of zinc deficiency the rat is readily able to accept and store normal amounts of vitamin A in the liver, but is unable to maintain normal levels of the vitamin in the plasma. We are attempting to determine whether the zinc functions only in allowing mobilization from the liver and subsequent transfer to the circulating blood, or whether it also influences in some way the actual utilization of the vitamin at the site of its function. Since some of the symptoms observed in vitamin A deficiency and zinc deficiency are quite similar the possibility must be considered that some of the symptoms accompanying zinc deficiency may actually be due to an ability to utilize the vitamin A, which may be in plentiful supply in the diet, and are therefore due to vitamin A deficiency. A factor which has complicated this study is the fact mobilization form the liver and maintenance of normal levels of vitamin A in the plasma appear to be dependent upon growth as well as upon the presence of zinc. It has been difficult to separate the effects of lack of growth and zinc deficiency since growth will not occur in the zinc deficient rat. We have observed however, that even when growth is prevented, the administration of zinc causes an increase in the retinol-binding protein (RBP) in the liver even though both RBP and vitamin A in plasma remain low until growth is permitted. We have used various means of restricting growth, such as reduced food intake, low protein diets and various B vitamin deficiencies, and in each case we have observed abnormally low levels of both vitamin A and zinc in the plasma even though excess amounts of both of these factors are administered either orally or by injection. Blood levels of both factors return to normal when growth is permitted by lifting the restriction. We are interested in determining why it appears so necessary for the young animal(less than adult) to be growing in order to maintain normal plasma levels of vitamin A and zinc, while an adult animal which has ceased growing apparently does not experience this difficulty. To determine if this is due to some physiological change with age we have maintained rats on diets sufficiently low in protein to cause abnormally low plasma levels of both vitamin A and zinc in young animals. When the animals are considered to be adult with respect to age we will determine their ability to maintain plasma vitamin A and zinc and the influence of allowing growth at that time.

The possibility was considered that newly formed cells may in some way be necessary for maintaining normal plasma vitamin A and zinc. We have subjected rats which were maintained at constant weight to repeated hemorrhage sufficient to cuase a severe anemia. These rats were then permitted to regenerate the blood cells. This should have resulted in a higher than normal percent of newly formed blood cells. This procedure did not increase the plasma levels of vitamin A even though excess A was given orally. The negative results with new blood cells should not necessarily eliminate the possibility that other types of newly formed cells may in some way have some effect.

In order to determine whether zinc is necessary for the actual utilization of vitamin A apart from its role in mobilization and transport, vitamin A is being injected directly into the blood of rats via the tail vein. If we are able by this means to maintain normal plasma A levels in the absence of zinc we may then be able to evaluate more precisely the invidivual roles of these two factors.

Protein has been considered to be a factor in maintaining the plasma vitamin A. By feeding controlled amounts of diets containing various levels of protein we have observed that the plasma A levels are related to the growth regardless of the amount of protein consumed. It was observed however that excessively high levels of protein appear to have an inhibiting effect on the maintenance of normal plasma A levels.

Keyword Descriptors: Nutrition; Vitamin A; Zinc; Germfree; Cycasin; Tumors.

Honors and Awards: NONE

Publications: NONE

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

A. The role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins. Investigation of the mechanism whereby hepatic plasma membranes selectively find circulating asialoglycoproteins as a prelude to macromolecular transport and lysosomal catabolism, may be summarized as follows.

1) The presumed role of glycosyltransferases in the specific recognition phenomena has been investigated utilizing a solubilized hepatic binding protein. Under optimally determined conditions, no transferase activity for sialic acid, galactose, N-acetylglycosamine, or fucose was detectable in a purified preparation which possessed a high specific activity for the binding of asialoglycoproteins.

2) Earlier estimates of the purity of the isolated binding protein were complicated by the extent of aggregation present in the final, water soluble preparation. This problem has been largely solved by the isolation of two non-identical subunits and the demonstration that the protein exists in aqueous solution as an oligomeric series with molecular weights ranging from 2.5×10^5 to greater than 1.5×10^6. The isolated, water soluble binding protein is a key intermediate for the projected study of the physiological significance of the binding reaction.

3) A rapid screening procedure has been developed for the recognition of abnormally high levels of circulating asialoglycoproteins in the serum of patients with varying pathologies. The amounts detectable are in the range of micrograms per ml of serum and the significance of the data obtained by this technique is being currently evaluated.

4) Examination of the subcellular particulate fractions of rat hepatocytes have revealed the binding protein to be present in cryptic form in the golgi and the lysosomes. The presence of the binding protein in such functionally diverse organelles has prompted an investigation of the role of this constituent in the biosynthesis and catabolism of the plasma proteins secreted by the liver.

B. The formation of the yeast primary septum. The yeast primary septum, a disk composed of chitin, has been used as a model for the study of morphogenesis at the molecular level:

1) A temperature-sensitive mutant was isolated, which is blocked in the cell cycle before septum formation. By allowing unbudded cells of the mutant to grow in a medium containing tritiated glucose it was shown that chitin synthesis starts early in budding. This synthesis is only required for the subsequent septum formation and not for budding itself, because wild-type cells can bud in the presence of sufficient concentrations of the antibiotic, Polyoxin, to completely inhibit chitin synthesis. In the presence of the brightener Calcofluor M2R, the cells budded in Polyoxin do not show an enhanced fluorescence at the mother cell-bud junction, which is prominent in control cells. It is concluded that the fluorescence is caused by chitin.

2. On the assumption that cells defective in septum formation will ultimately lyse, a simple method was devised for the screening of temperature-sensitive mutants which lyse at the non-permissive temperature. The method is based on locating the enzyme alkaline phosphatase, which is released by lysing cells, by the use of the chromogenic substrate, p-nitro phenylphosphate.

3. The protease (activating factor) which transform the chitin synthetase zymogen into the active form has been extensively purified. The major step in purification is an affinity chromatography in which the heat-stable proteinaceous inhibitor of the enzyme is coupled to a modified agarose.

4. By reinforcing the plasma membrane of yeast protoplasts with Concanavalin A before lysis, it has been possible to prepare the membranes essentially in a single piece. A purified membrane fraction obtained by gradient centrifugation was found to contain most of the chitin synthetase zymogen of the crude extract, thus confirming one of the major assumptions of our working hypothesis for septum formation.

Glycogen biosynthesis in yeast. In an effort to find a quick method for the purification of glycogen synthetase which would minimize proteolytic degradation of the enzyme, a procedure for the affinity chromatography of the enzyme on a glycogen-Concanavalin A-agarose column was developed.

C. Function of membrane components in bacteriocin tolerance. The goal of this project has been to study the structure, synthesis and function of a single membrane protein. The protein, TolG protein, is a major component of the outer membrane of gram negative bacteria and represents 16% of the total.

TolG protein has now been purified to the extent that it migrates as a single band in the two different gel electrophoresis systems.

The mutation in tolF mutants has been found to cotransduce with the pyrD locus. The tolF mutant may be phenotypically identical with the cmlB mutant, isolated resistant to chloramphenicol. Studies with F^1 episome show that both tolF and tolG mutations are recessive.

D. Biochemical Lesions in the Genetic Mucopolysaccharidoses.

A deficiency of iduronidase or sulfoiduronate sulfatase results in the Hurler or Hunter syndrome, respectively. The development of techniques for studying these enzymes is of importance for diagnostic purposes, and their purification may make possible their clinical application in enzyme replacement therapy.

Iduronidase is presently assayed by the colorimetric determination of phenol formed from the synthetic substrate, phenyl iduronide, which is difficult to prepare and is not readily available. A sensitive assay has been devised based upon the hydrolysis of tritium labeled anhydromannitol iduronide to form radioactive anhydrommanitol which is readily separated from the anionic substrate by electrophoresis or by means of ion-exchange chromatography. The labeled substrate is prepared from heparin.

E. Hormone-Dependent Differentiation of Mammary Gland. The study of the molecular and cytological phenomena involved in development of the mammary gland includes attempts to understand the mechanism of action of insulin, prolactin and placental lactogen.

1. Mammary epithelial cells from mature virgin and primiparous mice are very similar. Both are proliferatively and developmentally dormant, insulin--insensitive and non-secretory. Both can be converted into a secretory state in vitro. The only known difference between them is that cells from the virgin animal must necessarily traverse the cell-cycle as a prerequisite to differentiation. Cells from the primiparious animal do not have this requirement. It has been concluded from these and other observations that the two cell-types are arrested in different parts of the G_1 phase of the cell cycle. This may have some bearing on the epidemiological observation that early pregnancy in humans affords significant protection against mammary cancer.

2. It is commonly held that polypeptide hormones exert their characteristic biological effects by impinging on the plasma membrane, without the necessity fo entering the target cell. This question has been reexamined using nuclear preparations from mammary cells. A characteristic effect of prolactin and placental lactogen on intact mammary cells is enhancement of RNA synthesis. Surprisingly, these hormones accelerate RNA synthesis by the nuclear preparations and the effect is both cell and hormone specific.

3. Under conditions commonly employed with polypeptide hormone-Sepharose complexes, a form of insulin (ILM) could be solubilized from the complex that has greater biological activity on mammary cells than insulin itself. This may necessitate re-evaluation of certain other studies with hormone-Sepharose preparations in which it was assumed that the hormone remained covalently linked to Sepharose during the reaction with target cells.

The production of soluble super- active polypeptide hormones in this manner is not unique to insulin. Corresponding materials prepared from prolactin-Sepharose and placental lactogen-Sepharose have also been shown to have greater biological activity on mammary cells than the native hormones.

$S - O - \underset{NH}{C} - \overset{H}{N} -$ hormone, in which S = Sepharose. The release of soluble hormone-like material involves nucleophilic attack on the O - C bond by: $\overset{H}{\underset{H}{N}} - R$, generating $R \overset{H}{\underset{N}{\ }} - \underset{NH}{C} - \overset{H}{N} -$ hormone, the super-active hormone.

ILM has also been shown to have biological activity greater than native insulin when tested on other target cells. Although insulin itself does not enhance the rate of glucose oxidation by diaphragm from C57B1/6J $\overset{++}{ob}/\overset{++}{ob}$ mice, ILM does stimulate the oxidation. Furthermore adipose tissue from these obese mice is about 50 times as sensitive to ILM in terms of glucose oxidation as it is to native insulin. With corresponding tissues from lean

C57Bl mice, ILM and insulin are equipotent. It is apparent that any palliative advantage of ILM would be on tissues which are resistant to native insulin.

F. Hormonal Interaction on the Biosynthesis of Spermidine during Differentiation of Mouse Mammary Epithelium in vitro. Terminal differentiation of mouse mammary epithelium is elicited in vitro in the presence of insulin, glucocorticoid and prolactin. Recently we presented several lines of evidence to indicate that a polyamine, spermidine, is involved in milk protein synthesis during this differentiation process. The present study has been undertaken to elucidate the mechanism whereby the interplay of hormones enhances the biosynthesis of spermidine in cultured mammary epithelium. Emphasis is placed on the effects of hormones on the activities of several enzymes which are required for spermidine formation. The biosynthesis of spermidine involves the following steps: Arginine → ornithine → putrescine → spermidine. The data show that prolactin, in the presence of insulin, causes a several-fold increase in the activity of arginase, an enzyme which catalyzes the formation of ornithine. The activity of ornithine decarboxylase, which catalyzes the formation of putrescine from ornithine (step 2), is controlled by several factors. First, the activity increases very rapidly by simply incubating the tissue explants in organ culture medium. The increase occurs as early as one hour after the incubation, and reaches the maximum at 3 - 4 hours. Secondly, addition of insulin augments the increase in the enzyme activity. Thirdly, prolactin, in the presence of insulin and/or glucocorticoid enhances the enzyme activity. In all cases, the increase in the enzyme activity is completely blocked by inhibition of protein synthesis, but only partially blocked by inhibition of RNA synthesis. Moreover, addition of cyclic AMP derivatives, but not cyclic GMP derivatives, causes enhancement of the enzyme activity. During the first few hours, the activities of adenyl cyclase and phosphodiestrase increase rapidly, with a net reduction of cellular cyclic AMP level. Since the effect of cyclic AMP is additive to that of insulin and prolactin, it appears tht the mode of action of cyclic AMP is different from those of insulin and prolactin. Glucocorticoid in combination with insulin increases the activity of S-adenosyl-L-methionine decarboxylase, which is involved in the final step of spermidine formation. These observations indicate that insulin, glucocorticoid and prolactin interact to stimulate a group of enzymes that are involved in the biosynthesis of spermidine. In view of our previous observation that spermidine is necessary for milk protein synthesis, such hormonal interplay may represent a fundamental mechanism for the regulation of lactogenesis in the development of mammary gland.

G. Differentiation of Lymphoid Cells. Attempts are being made to analyze thecellular events which are prerequisite for the induction of the synthesis and secretion of immunoglobulins with particular emphasis on the conversion of precursors of immunoglobulin-producers (B cells) and on the role of cell-free mediators derived from T cells (thymus-derived lymphocytes) in cellular cooperation.

Rabbit spleen cells when cultured in vitro for 72 hours undergo differentiation into IgM-producing cells in the absence of added antigen. This spontaneous IgM production requires the participation of T cells in the sense that the treatment of spleen cells with anti-thymocyte serum (ATS) inhibits the response, and addition of thymocytes restores it. Furthermore, addition of thymocytes to untreated spleen cells causes enhancement of IgM production.

Thymocytes culture filtrate (Fil) and thymocyte extract (Ext) were found to possess an enhancing activity on IgM production of spleen cells, an effect which appears to occur at the same site for both factors.

Other evidence indicates that Fil and Ext cannot completely substitute for live T cells and that their function represents only a part of the multiple function of T cells as a "helper" of immunoglobulin production by B cells.

Incubation of spleen cells with Fil or Ext for a short period during the course of tissue culture revealed that the cells are insensitive to the enhancing stimulation during the first 24 hours and that most cells are sensitive between 36 to 60 hours. It was also shown that DNA synthesis precedes this stage. Furthermore, Fil and Ext were shown to have no mitogenic activities. They could exert their enhancing effect even when added together with mitotic inhibitor (hydroxyurea) at 48 hours, if DNA synthesis was allowed before the addition. These findings suggest that the target cells which are most susceptible to stimulation are those which have already experienced cell division, and enhancement by Fil and Ext does not involve further cell division.

H. Glutathione S-transferases, a Family of Catalytically Active Binding Proteins. The glutathione S-transferases are among the major detoxication systems of the animal kingdom and perform this function by related mechanisms which all involve binding of protein and ligand. Work with these enzymes has provided five homogeneous transferases from human liver and five homogeneous transferases from rat liver. The availability of clean systems has allowed extensive studies on the chemical and kinetic mechanisms of the enzymatic reaction as well as the specificity of binding.

The available data are consistent with the concept of the glutathione S-transferases as binding proteins. Once bound, a ligand may follow three paths. In the simplest case, ligands such as bilirubin or indocyanin green are reversibly bound; the protein serves a storage function in liver prior to further metabolism or excretion of the ligand into bile. In the second case, the ligand is a compound with a sufficiently electrophilic center that the other substrate of the enzyme, the nucleophilic glutathione thiolate bound in close juxtaposition, is able to react with it. Here, the protein detoxified by performing as an enzyme with substrates which include such disparate functional groups as are present in nitroglycerin, ethacrynic acid, arene oxides and other epoxides, halogen and nitro derivatives of both alkyl and aromatic nuclei and many others. In the third case, the ligand is sufficiently reactive that it can form covalent bands with the protein itself; in effect, the ligand serves as an active-site reagent as is found with certain carcinogens or, as a model compound in our hands, 1-chloro-2,4-dinitrobenzene. Activity of the last sort leads to destruction of the protein.

Since this group of enzymes comprise 10% of the extractable protein from rat liver and 2% from human liver, it is clear that sufficient material is available for all three detoxication functions.

I. Investigations on the Biochemical Abnormality Underlying Human Cystinosis. Human cystinosis is a rare and often fatal inherited disease characterized by excessive intralysosomal storage of the amino acid cystine in certain cells of the body.

The level of intracellular free cystine in cystinotic cells is markedly sensitive to small increases in incubation temperature. For example, cells incubated at 39° for two days lose one-half to two-thirds of their cystine content as compared with control cells maintained at 37°. There is no indication of cell damage as a result of the higher temperature as determined by several parameters of cell viability. Furthermore, the effect of temperature was completely reversible: cells which had been exposed to the higher temperature for 2 - 4 days and subsequently returned to their former condition at 37° regained their original intracellular content of cystine.

The effect of the pH of incubation medium on intracellular cystine content of cystinotic cells varied. One cell line exhibited markedly increased intracellular levels (2 - 4 fold) of cystine as the pH was increased from 6.8 to 8.0; this effect was reversible.

Protein-free extracts from normal cystinotic cells exposed to 35S-cystine showed a prominent peak of radioactivity corresponding to reduced glutathione and minor peaks corresponding to oxidized glutathione and an unidentified component with positive mobility intermediate between cystine and cysteamine. Cystinotic extracts showed, in addition, a large peak of cystine but no other unique area of radioactivity. These results are consistent with the idea that the elevated levels of cystine in cystinotic cells are not the consequence of blockage in the catabolic pathway of cystine at a point one or more steps removed from the amino acid itself.

J. Studies on Naturally Occurring Sulfur Nucleotides.

1. Enzyme-Polynucleotide Interactions. Factor A, the first enzyme in 4-thiouridine biosynthesis, was shown to be eluted from cellulose phosphate by tRNA or Poly U, but not by Poly A, Poly C, UMP, UTP, uridine or 4-thioUMP. This list has now been extended to show that Poly dU will elute the enzyme, but not Poly dT, U(pU) 11-19, dpT)12-18, AMP, ATP, or dATP. The tRNA-Factor A complex can also be demonstrated electrophoretically. Thus we conclude that for formation of the enzyme complex, 1) a chain length above 20 is required, 2) the presence of deoxyribose has no effect, 3) the 5-CH_3 substituent of thymine polymers blocks binding, and 4) secondary structure of the polynucleotide is not required, since Poly U has none under these conditions.

2. Competition between Poly U and tRNA. Poly U itself is not a sulfur acceptor, but it is unclear whether this represents a specificity of Factor A or of the second enzyme. Poly U will inhibit the action of Factor A on tRNA. The inhibition is roughly linear with increasing Poly U concentration, but only about 20% depression is seen when the ratio of U chains to

tRNA molecules is 60 to 1. The presence of Poly U does not affect the activity of the second enzyme toward activated tRNA.

K. Thermodynamic and Kinetic Studies of Protein Structure. Proteins demonstrate great specificity in their behaviour, both in the way that the newly synthesized poly peptide chain can fold into the "native" confirmation and this form shows its enzymic activity. Kinetic studies on these processes may reveal some of the physical principles underlining these phenomena. It has been shown for example, that inhibitors of pepsin activity also inhibit the unimolecular activation of pepsinogen. This indicates that contrary to previous reports this activation is an enzymic reaction. Using isoamyl alcohol, a good inhibitor of pepsin, it has been possible to activate pepsinogen with the loss of only one activation peptide, instead of the six or seven normally found.

L. Particulate Enzymes of Carbohydrate Metabolism. This project is intended as a study of the occurence, properties and functions of some of the membrane-bound enzymes liver and kidney which catalyze the synthesis, hydrolysis and transport of a variety of phosphorylated sugars and sugar alcohols. Of particular interest is the microsomal enzyme glucose-6-phosphatase which, in addition to its well-known hydrolytic function, has been found to be capable of catalyzing synthetic reactions utilizing such high energy compounds as inorganic pyrophosphate and carbamyl phosphate. The quantitative levels of these membrane-bound enzymes have been found to be particularly susceptible in vivo to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, cortisone and insulin administration.

Current studies are concerned with the development of glucose-6-phosphatase and its related enzyme activities in the endoplasmic reticulum of the livers and kidneys of young rats in the hope of contributing to an understanding of the mechanism of the synthesis of biological membranes. Glucose-6-phosphatase is known to make its appearance at birth and to undergo a dramatic increase in relative specific activity during the first four days of life, followed by a gradual decrease to adult levels of activity. The enzyme has been shown to have a significant component of "latent" enzyme activity, which is apparent only after pretreatment of the membranes with detergents or controlled alkaline conditions. In contrast to earlier studies, the enzyme levels of both rough and smooth subfractions of the membranes are being tested as a function of the age of the animals, before and after the enzymes have been fully activated in vitro. So far, it has been found that the postnatal "overshoot" in enzyme activity observed in unfractionated microsomes is largely a property of the rough membrane fraction. The enzyme in the smooth subfraction increases more gradually with increasing age. The phenomenon of latency has been found to be quantitatively more significant in very young than in older animals.

The hepatopancreas of Limulus (Xiphosura polyphemus) has an enzyme complex with many properties very similar to those of the liver glucose-6-phosphatase of rat, man and other high vertebrates. The hydrolytic and phosphotransferase activities also have an organ distribution comparable to vertebrates, since they occurred not only in the "liver" but also in the intestinal mucosa and the coxal gland, thought to correspond to the kidney of higher forms. In the organs of other invertebrate animals studied, the comparable enzyme activities either had different properties or were entirely absent. Limulus is a species which has in the past few years attracted the attention of the medical profession because of the increasing use of a preparation made from its circulating blood cells in a very sensitive test for bacterial endotoxins.

M. Phospholipid Stimulation of Glucose 6-Phosphate Formation in Brain. Unsupplemented isolated brain fractions are capable of synthesizing glucose 6-phosphate by an unknown pathway which can be stimulated by phospholipids. Glucose 6-phosphate formation was first observed in this laboratory as a concomitant of phosphatidyl inositol breakdown in brain homogenate stimulated by deoxycholate. From this circumstance and the similarity in kinetics of formation and yield between water soluble products of phosphatidyl inositol degradation and glucose 6-phosphate, it was tentagively concluded that glucose 6-phosphate was derived from a novel phospholipid of brain similar to those recently described in bacteria. Supporting this hypothesis was the finding that whole brain lipid could replace deoxycholate in stimularing brain low-speed supernatant to produce glucose 6-phosphate. On fractionation of the lipid, however, it was found that ordinary phospholipids like phosphatidyl choline and phosphatidyl ethanolamine could replace the whole lipid. Clearly the phospholipid was not a substrate but a stimulant and the search for the substrate was directed elsewhere. Addition of various inhibitors has failed to implicate known pathways of glucose 6-phosphate formation, since none of them has inhibited formation of the ester. Glucose-^{14}C failed to incorporate radioactivity into glucose 6-phosphate. Protein-free extracts of brain added to dialyzed low-speed supernatant serve as sources of phospholipid-stimulated glucose 6-phosphate and/or of a cofactor required for the reaction.

Project No. Z01 AM 17001-09 LBM
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins.

Previous Serial Number: NIAMDD-LBM-1

Principal Investigator: Gilbert Ashwell

Other Investigators: Jean Hickman, Research Chemist
Toshisuke Kawasaki, Visiting Fellow
William Pricer, Research Chemist
Joan Lunney, Post Graduate Student

Cooperating Units: None

Man Years

Total: 3
Professional: 3
Other:

Project Description:

The major objective of this project has been to investigate the nature of the mechanism whereby hepatic plasma membranes selectively find circulating asialoglycoproteins as a prelude to macromolecular transport and lysosomal catabolism.

Hepatic plasma membranes, as well as other intracellular organelles, and intact, viable hepatocytes are isolated by conventional methods of cell fractionation and their binding properties studied with ^{125}I-asialo-orosomucoid as the principal ligand. Solubilization and purification of the binding protein is accomplished by detergent extraction and affinity chromotography.

The results of continuing studies on the chemical and biological properties of the hepatic membrane protein responsible for the binding of desialylated plasma proteins may be summarized as follows:

1) The presumed role of glycosyltransferases in the specific recognition phenomena has been investigated utilizing a solubilized hepatic binding protein. Under optimally determined conditions, no transferase activity for sialic acid, galactose, N-acetylglycosamine, or fucose was detectable in a

purified preparation which possessed a high specific activity for the binding of asialoglycoproteins.

2) Earlier estimates of the purity of the isolated binding protein were complicated by the extent of aggregation present in the final, water soluble preparation. This problem has been largely solved by the isolation of two non-identical subunits and the demonstration that the protein exists in aqueous solution as an oligomeric series with molecular weights ranging from 2.5 x 10^5 to greater than 1.5 x 10^6. (T. Kawasaki)

3) A rapid screening procedure has been developed for the recognition of abnormally high levels of circulating asialoglycoproteins in the serum of patients with varying pathologies. The amounts detectable are in the range of micrograms per ml of serum and the significance of the data obtained by this technique is being currently evaluated. (J. Lunney)

4) Examination of the subcellular particulate fractions of rat hepatocytes have revealed the binding protein to be present in cryptic form in the golgi and the lysosomes. The presence of the binding protein in such functionally diverse organelles has prompted an investigation of the role of this constituent in the biosynthesis and catabolism of the plasma proteins secreted by the liver. (W. Pricer and J. Hickman)

The isolated, water soluble binding protein is a key intermediate for the projected study of the physiological significance of the binding reaction. For this purpose, a high titer antibody has been produced in goats and will be used to monitor the rate and extent of the binding reaction in vivo. In addition, the availability of the purified binding protein will provide a means for examining in detail the postulated role for glycosyl transferases in the finding reaction.

Studies with intact hepatocytes will be continued in an attempt to clarify the mechanism whereby the binding protein participates in the recognition as well as the uptake and intracellular transport of the ingested material.

Keyword Descriptors:

Serum glycoprotein, asialoglycoprotein, hepatic plasma membrane, hepatic binding protein, galactose, sialic acid, neuraminidase

Honors and Awards:

None

Publications:

Ashwell, G. and Morell, A.G.: The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Advances in Enzymology* 41: 99-128, 1974.

Hudgin, R. L., Pricer, W.E., Ashwell, G., Stockert, R.J. and Morell, A.G.: The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. *J. Biol. Chem.*, 249: 5536-5543, 1974.

Pricer, W.E., Hudgin, R. G., Ashwell, G., Stockert, R.J. and Morell, A.G.: A membrane receptor protein for asialoglycoproteins. In Jakoby, W.B. and Wilchek, W. (eds.): *Methods in Enzymology*, vol. XXXIV. New York, Academic Press, 1974, pp. 688-691.

Hudgin, R.L. and Ashwell, G.: Studies on the role of glycosyltransferases in the hepatic binding of asialoglycoproteins. *J. Biol. Chem.*, 7369-7372, 1974.

Lunney, J. and Ashwell, G.: The effect of phospholipase on the binding of asialoglycoproteins by rat liver plasma membranes. *Biochim. Biophys. Acta*, 367: 304-315, 1974.

Hickman, J. and Ashwell, G.: Studies on the hepatic binding of asialoglycoproteins by hepatoma tissue and by isolated hepatocytes. In Tager, J.M., Hooghwinkel, G.J.M. and Daems, W.Th. (eds.): *Enzyme Therapy in Lysosomal Storage Diseases*. The Netherlands, North Holland, 1974, pp. 169-172.

Ashwell, G. and Morell, A.: The dual role of sialic acid in the hepatic recognition and catabolism of serum glycoproteins. *Biochem. Soc. Symp*. 40: 117-124, 1974.

Ashwell, G.: The role of cell surface carbohydrates in binding phenomena. In Jamieson, A. and Robertson, D.M. (eds.): *Mammalian Cell Membranes*. vol. 4, London, Butterworths, in press.

Project No. Z01 AM 17002-05 LBM
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Glutathione transferases as catalysts and binding proteins

Previous Serial Number: NIAMDD-LBM-2

Principal Investigator: William B. Jakoby, Research Chemist

Other Investigators: William H. Habig, Staff Fellow
Jeanne N. Ketley, Staff Fellow
James M. Keen, Graduate Student
(Cornell U.)

Cooperating Units: None

Man Years:

Total:	5
Professional:	4
Others:	1

Project Description:

The glutathione S-transferases are among the major detoxication systems of the animal kingdom and perform this function by related mechanisms which all involve binding of protein and ligand. Our work with these enzymes has provided five homogeneous transferases from human liver and five homogeneous transferases from rat liver. The availability of clean systems has allowed extensive studies on the chemical and kinetic mechanisms of the enzymatic reaction as well as the specificity of binding.

The available data are consistent with the concept of the glutathione S-transferases as binding proteins. Once bound, a ligand may follow three paths. In the simplest case, ligands such as bilirubin or indocyanin green are reversibly bound; the protein serves a storage function in liver prior to further metabolism or excretion of the ligand into bile. In the second case, the ligand is a compound with a sufficiently electrophilic center that the other substrate of the enzyme, the nucleophilic glutathione thiolate bound in close juxtaposition, is able to react with it. Here, the protein detoxifies by performing as an enzyme with substrates which include such disparate functional groups as are present in nitroglycerin, ethacrynic acid, arene oxides and other epoxides, halogen and nitro derivatives of both alkyl and aromatic nuclei and many others. In the third case, the ligand is sufficiently reactive that it can form covalent bands with the protein itself; in effect, the ligand serves as an active-site reagent as is found with certain carcinogens or, as a model compound in our hands, 1-chloro-2,4-dinitrobenzene. Activity of the last sort leads to destruction of the protein.

Since this group of enzymes comprise 10% of the extractable protein from rat liver and 2% from human liver, it is clear that sufficient material is available for all three detoxication functions.

Work on glutathione conjugation by enzymes from liver is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxication of noxious compounds. It is expected that investigations dealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

Keyword Descriptors:

Glutathione S-transferases; detoxication; ligandin; carcinogen detoxication, bilirubin, binding proteins

Honors and Awards:

None

Publications:

Jakoby, W. B. and Wilchek, M. (Eds.): Affinity Techniques Enzyme Purification: Part B. Academic Press, 1974, 810 pp.

Wilchek, M. and Jakoby, W. B.: The literature on affinity chromatography. In Jakoby, W. B. and Wilchek, M. (Eds.): Methods in Enzymology. New York, Academic Press, 1974, pp. 3-10.

Habig, W. H., Pabst, M. J. and Jakoby, W. B.: Glutathione S-transferases; the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139, 1974.

Pabst, M. J., Habig, W. H. and Jakoby, W. B.: Glutathione S-transferase A; a novel kinetic mechanism in which the major reaction pathway depends on substrate concentration. J. Biol. Chem. 249:7140-7150, 1974.

Habig, W. H., Pabst, M. J., Fleischner, G., Gatmaitan, Z., Arias, I. M. and Jakoby, W. B.: The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proc. Nat. Acad. Sci., USA 71: 3879-3882, 1974.

Packman, P. M. and Jakoby, W. B.: 5-Phospho-α-D-ribose-1-diphosphate. In Bergmeyer, H. V. (Ed.): Methods of Enzymatic Analysis. New York, Academic Press, 1974, pp. 1346-1349.

Kohn, L. D., Hurlbert, R. E. and Jakoby, W. B.: L(+)-Tartrate and meso-Tartrate. In Bergmeyer, H. V. (Ed.): *Methods of Enzymatic Analysis*. New York, Academic Press, 1974, pp. 1397-1403.

Jakoby, W. B.: Oxalate. In Bergmeyer, H. V. (Ed.): *Methods of Enzymatic Analysis*. New York, Academic Press, 1974, pp. 1542-1545.

Scher, W. and Jakoby, W. B.: Maleate. In Bergmeyer, H. V. (Ed.): Methods of Enzymatic Analysis. New York, Academic Press, 1974, pp. 1622-1624.

Myers, J. S. and Jakoby, W. B.: Glycerol as an agent eliciting small conformational changes in alcohol dehydrogenase. *J. Biol. Chem*. 250, in press, 1975.

Habig, W. H., Keen, J. M. and Jakoby, W. B.: Glutathione S-transferase in the formation of cyanide from organic thiocyonates as an organic nitrate reductase. *Biochem. Biophys. Res. Comm.*, in press, 1975.

Jakoby, W. B.: Glutathione S-transferases. A family of binding proteins which includes ligandin. In Berlin, N. I., Berk, P. D. and Watson, C. J. (Eds.) Fogarty Symposium on Bile Pigments. Washington, D. C., U. S. Government Printing Office, 1975, in press.

Bradbury, S., Clark, J. F., Steinman, C.R. and Jakoby, W.B.: Aldehyde dehydrogenase from yeast. *Methods Enzymology* 41: 354-360, 1975.

Project No. Z01 AM 17003-08 LBM
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Polysaccharides in Morphogenesis and in the Storage of Energy

Previous Serial Number: NIAMDD-LBM-5

Principal Investigator: Enrico Cabib, Research Chemist

Other Investigators: Rodney Ulane, Staff Fellow
James Braatz, Staff Fellow
Angel Duran, Guestworker
Sherwin Kane, Post Doctoral Fellow

Cooperating Units: Blair Bowers, NHLI

Man Years

Total:	5
Professional:	5
Other:	0

Project Description:

Two different projects are carried out in this laboratory, one on the formation of the yeast primary septum and the other on glycogen synthesis in yeast. They will be treated separately in that sequence.

The formation of the yeast primary septum. The yeast primary septum has been chosen as a model for the study of morphogenesis at the molecular level, because of its simple shape and chemical composition, that of a disk consisting of the polysaccharide chitin. More information of a biochemical nature is available for this system than for any other morphogenetic model, thanks to the studies carried out in this laboratory. It is hoped that the results obtained in the course of these investigations will suggest general mechanisms utilized by the cell in the formation of specific shapes.

1. The timing of chitin synthesis during the cell cycle has been studied with different approaches. One of them entailed the use of a mutant, isolated in our laboratory, which is blocked in the cell cycle before septum formation. When unbudded cells of this mutant are allowed to grow at the non-permissive temperature, they all bud, but stop development

when the bud has the same site as the mother cell, before the septum is made. By using tritiated glucose in the medium, it could be shown that a substantial synthesis of chitin takes place during this period. It is concluded that chitin starts accumulating at the neck between mother cell and bud at an early time in the cell cycle, before it finally grows centripetally to close the connection between the two cells.

Whether this early formation of chitin is necessary for the process of bud emergence was investigated by the use of the antibiotic Polyoxin D, which was shown in previous studies to inhibit chitin synthesis and primary septum formation. Unbudded cells of a wild type yeast were all able to bud in the presence of a concentration of Polyoxin D which was sufficient to totally inhibit chitin synthesis, as verified by incorporation experiments using tritiated glucose in the medium. Most of the cells became aberrant at a later stage, when the bud approached the size of the mother cell. It is concluded that chitin synthesis, although initiated in early budding is only required for septum formation.

Previous workers had observed the appearance of a marked fluorescence at the bud scar site or at the neck between mother cell and bud, in the presence of certain dyes. The fluorescence has been attributed by different authors to either chitin or glucan. This phenomenon was reinvestigated using Calcofluor White M2R new as the fluorescent compound. A bright ring at the junction between mother cell and bud was indeed observed even in very small buds, but it was completely absent in cells allowed to bud in the presence of Polyoxin D. Therefore it appears that the bright ring is indeed due to the presence of chitin. This finding may provide a much-needed method for the screening of mutants defective in chitin synthesis.

2. As mentioned above, it is important for the understanding of the mechanism of septum formation, to obtain mutants defective in chitin synthesis. It was argued that such mutants, which would have to be conditional - lethal, i.e. temperature - sensitive, should show the same phenotype at the non-permissive temperature as the wild type does under conditions in which chitin is not synthesized. From the experiments with Polyoxin D it was known that inhibition of chitin synthesis finally leads to the lysis of a significant percentage of the cell in the culture. Therefore, it was decided to use lysis at the non-permissive temperature as a first screening method for mutants. Accordingly, a simple procedure was developed for the detection of colonies containing lysed cells, as follows. After mutagenesis and growth at 25°, the colonies are exposed to 37° overnight and the Petri dishes are overlaid with a solution containing dilute agar, p-nitrophenol phosphate and an alkaline buffer. Lysed cells liberate the intracellular alkaline phosphatase, which causes the formation of p-nitrophenol. The corresponding colonies can be detected because of their yellow color and halo. Cells are then picked up with a loop, observed under the microscope, and those with the desired morphology are recovered. In this way, several mutants were obtained, which are now under study. Some of these mutants appear to be blocked at a stage previous to septum formation, and one

of them (L-2-42) has already been used in experiments described in the previous section.

3. The protease (activating factor) which transforms the chitin synthetase zymogen into the active form has been extensively purified. The enzyme is solubilized by sonication of a particulate fraction after lysis of yeast protoplasts. The following step is chromatography on a DEAE-cellulose column. Finally, an affinity chromatography is carried out, where the solid phase is a modified agarose, to which the purified proteinaceous inhibitor of the activating factor has been attached. Upon passing through this column, the activating factor binds to the inhibitor, and it is later eluted by washing with 6M urea at pH 4.6. The overall purification in this procedure is about 200-fold, which is rather high, considering that the particulate starting material was already devoid of all the soluble proteins of the cell. The ratio of zymogen-activating and proteolytic activities remained constant throughout the purification.

The purified enzyme has unusual properties, in that it does not yield stainable bands after disc gel electrophoresis at pH 8.5 either in the absence or in the presence of urea or of sodium dodecylsulfate. However, at acidic pH in the presence of 4 M urea two bands of very dissimilar mobility were observed. Because it would be a strange coincidence that two proteins with such uncommon properties have been purified together, it seems probable that the two bands are subunits originated from the same protein.

The molecular and kinetic properties of the protease are now under study.

4. In a working hypothesis we presented in 1971 it was proposed that the activating factor of chitin synthetase would be enclosed in vesicles, whereas the zymogen would be attached to the plasma membrane. By directing the protease-containing vesicles to a specific spot of the plasma membrane, localization of zymogen activation, hence of chitin synthesis, would be obtained. Part of this hypothesis was confirmed when we were able to isolate from yeast a vacuole fraction which contained the activating factor. It remained to be verified whether the zymogen was indeed attached to the plasmalemma. Although several preparations of yeast plasmalemma have been described in the literature, they all lead to the isolation of small membrane fragments. In the absence of a specific label or marker, it is difficult to exclude that these preparations are mixtures of external and internal membranes. For these reasons attempts were made in our laboratory to obtain membranes in large, easily identifiable pieces. Partial success was obtained by cross-linking the membrane with glutaraldehyde before cell disruption, but when the apparently large fragments obtained were observed in the election microscope, they were seen to consist of a myriad of vesicles somehow attached to each other and including other material. This vesiculation probably occurred as a consequence of the strongly alkaline conditions used in lysing the glutaraldehyde-fixed protoplasts.

Recently, a method utilized by Scarborough for the isolation of Neurospora membranes was applied in a modified form to yeast protoplasts. This procedure consists in reinforcing the protoplast membrane by coating it with Concanavalin A previous to lysis, whereby the membranes remain essentially in one piece. By centrifugation on a linear gradient of renografin it was possible to recover the membranes in good yield. Of the total chitin synthetase activity recovered in the gradient, about 80% was associated with the membrane band, with a three-fold purification with respect to protein. The membranes, as observed by light or electron microscopy, are about 5-6 microns in length or width, the approximate dimensions of an intact cell.

Another major prediction of our working hypothesis has thus been verified. This finding will also make possible a thorough chemical and enzymatic study of the yeast plasmalemma.

Glycogen biosynthesis in yeast. The immediate goal of our work in this area was to compare the glycogen synthetase from wild type yeast with that of the "glycogenless" mutant GS-36, in order to ascertain whether the different behavior of the two strain could be attributed to a difference in the enzymes. Previous work in collaboration with K.-P. Huang had shown that glycogen synthetase is exceedingly sensitive to the proteases contained in yeast extracts and that proteolytic action led to an increase in the glycogen synthetase activity measured in the absence of glucose-6-phosphate. Thus, a meaningful comparison of the enzymes from the two strains required a method of purification which would avoid artifactual proteolytic changes. In addition to using protease inhibitors in the initial steps, it was deemed important to have a step early in purification which would separate glycogen synthetase from proteases. For this purpose, an affinity chromatography procedure was developed, in which the enzyme is absorbed to a glycogen-Concanavalin A-agarose column and later eluted with α-methyl mannoside. By further fractionation with DEAE-cellulose and Sephadex G-200 columns, an enzyme was obtained which compares in specific acitivity with that prepared by the older procedure. However, some impurities were detected by disc gel electrophoresis of the purified enzyme. Further studies in this direction are in progress.

Keyword Descriptors:

yeast, septum, chitin, budding, protease, morphogenesis, affinity chromatography, lysis mutants, glycogen, glycogen synthetase

Honors and Awards:

None

Publications:

Cabib, E., Ulane, R., Bowers, B. A Molecular Model for Morphogenesis: The Primary Septum of Yeast. Current Topics, Vol. 8: 1-30, 1974.

Bowers, B., Levin, G., and Cabib, E. Effect of Polyoxin D on Chitin Synthesis and Septum Formation in Saccharomyces cerevisiae. J. of Bact., Vol. 119: 564-575, 1974.

Huang, K. P. and Cabib, E. Yeast Glycogen Synthetase in the Glucose 6-Phosphate-dependent Form, I. J. Biol. Chem., Vol. 249: 3851-3857, 1974.

Huang, K. P. and Cabib, E. Yeast Glycogen Synthetase in the Glucose 6-Phosphate-dependent Form, II. J. Biol. Chem., Vol. 249: 3858-3861, 1974.

Ulane, R. E. and Cabib, E. The Activating System of Chitin Synthetase from Saccharomyces cerevisiae. J. Biol. Chem., Vol. 249: 3418-3422, 1974.

Project No. Z01 AM 17004-07 LBM
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Thermodynamic and Kinetic Studies of Protein Structure

Previous Serial Number: NIAMDD LBM 6

Principal Investigator: Peter McPhie

Other Investigators: Robert Scruggs

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 1 1/4
Others: none

Project Description:

Objectives

Proteins demonstrate great specificity in their behaviour, both in the way that the newly synthesized poly peptide chain can fold into the "native" confirmation and this form shows its enzymic activity. Kinetic studies on these processes may reveal some of the physical principles underlining these phenomena.

Methods Employed

Visible and ultra violet spectroscopy; rapid kinetic experiment in Aminco T. jump and Aminco-Morton stop flow apparatus. These are being equipped with a "Dasar" data collection and processing system which should make the acquisition of results both quicker and more accurate.

Major Findings

It has been shown that inhibitors of pepsin activity also inhibit the unimolecular activation of pepsinogen. This indicates that contrary to previous reports this activation is an enzymic reaction. Using iso. amyl alcohol, a good inhibitor of pepsin, it has been possible to activate pepsinogen with the loss of only one activation peptide, instead of the six or seven normally found.

Proposed course of Study

Studies on the activation of pepsinogen are being continued. It is hoped to learn more on the mechanism of this reaction by the effect of modification of functional groups on the reaction, by the interaction of the homogeneous activation peptide with pepsin and by comparative studies on the confirmations of pepsin and pepsinogen.

Kinetic studies on the folding of globular proteins have revealed the presence of transient intermediates in the reaction. Their short life times and low concentrations have made studies of their nature difficult. However partially unfolded forms of proteins may be obtained under various conditions. Studies on the transitions between these various forms and the native proteins should show if they are analogous to the intermediates mentioned previously.

Keywork Descriptors:

Protein folding; Zymogen activation; Pepsinogen; Kinetics

Honors and Awards:

None

Publications:

Miles, E. W. and McPhie, P.: Evidence for a rate determining proton abstraction in the serive deaminase reaction of the B_2 subunit of trytophan synthetase. J. Biol. Chem., 249: 2852-2857, 1974.

Chou, J. Y., Singer, M. F. and McPhie, P.: Kinetic Studies on the phosphorolysis of polynucleotides of polynucleotide phosphorylase. J. Biol. Chem., 250: 508-514, 1975.

Klee, C. B., Kirk, K. L., Cohen, L. A. and McPhie, P.: Histidine ammonia lyase, the use of 4-fluorohistidine in identification of the rate determining step. J. Biol. Chem., in press.

Project No. Z01 AM 17005-10 LBM
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

Previous Serial Number: NIAMDD-LBM-4

Principal Investigator: Marie N. Lipsett

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1.1
Professional: 1.0
Other: 0.1

Project Description:

Objectives

1. To explore the two-enzyme sulfurtransferase system which forms 4-thiouridine in tRNA.
2. To determine, if possible, the role of 4-thiouridine in the tRNA molecule.

Methods Employed

Standard methods were used for separations involving proteins or nucleic acids, i.e., salt fractionation, phase partition, column chromatography and electrophoresis.

Major Findings

1. Enzyme-Polynucleotide Interactions. Factor A, the first enzyme in 4-thiouridine biosynthesis, was previously shown to be eluted from cellulose phosphate by tRNA or Poly U, but not by Poly A, Poly C, UMP, UTP, uridine or 4-thioUMP. This list has been extended to show that Poly dU will elute the enzyme, but not Poly dT, $U(pU)_{11-19}$, $(d_pT)_{12-18}$, AMP, ATP, or dATP. The tRNA-Factor A complex can also be demonstrated electrophoretically. Thus we

conclude that for formation of the enzyme complex, 1) a chain length above 20 is required, 2) the presence of deoxyribose has no effect, 3) the 5-CH_3 substituent of thymine polymers blocks binding, and 4) secondary structure of the polynucleotide is not required, since Poly U has none under these conditions.

2. Competition between Poly U and tRNA. Poly U itself is not a sulfur acceptor, but is is unclear whether this represents a specificity of Factor A or of the second enzyme. Poly U will inhibit the action of Factor A on tRNA. The inhibition is roughly linear with increasing Poly U concentration, but only about 20% depression is seen when the ratio of U chains to tRNA molecules is 60 to 1. The presence of Poly U does not affect the activity of the second enzyme toward activated tRNA.

Proposed course of project

The complexes between Factor A and either tRNA or Poly U will be used as substrates to study the modification introduced by the addition of ATP to the reaction. This is the modified intermediate which in turn is thiolated by the second enzyme in the presence of cysteine.

A series of F-prime strains of E. coli K_{12} has been obtained which contain known portions of the E. coli chromosome duplicated on the episomes. These strains will be screened to locate the one(s) having a double level of either or both of the transthiolating enzymes. Once the approximate location of these enzymes is established on the chromosome, known deletion mutants in this area will be examined to find a strain lacking the enzyme and the ability to thiolate tRNA. This will give us another approach to the role of 4-thiouridine in tRNA.

Keyword Descriptors:

tRNA, thionucleotide, transthiolation

Honors and Awards:

None

Publications:

None

Project No. Z01 AM 17006-01 LBM
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
Nov. 1, 1974 through June 30, 1975

Project Title: Function of membrane components in bacteriocin tolerance

Previous Serial Number: None

Principal Investigator: John F. Foulds

Other Investigators: Tuu-jyi Chai

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Others:	

Project Description:

Objectives:

The goal of the research project has been to study the structure, synthesis and function of a single membrane protein. The protein to be studied, the TolG protein, is a major component of the outer membrane of gram negative bacteria representing 16% of the total protein of this fraction.

We hope to determine the role of the TolG protein in the functional organization of the outer membrane and in bacteriocin sensitivity. Determination of the chemical composition and site of synthesis of the protein will be the initial steps toward this goal. We hope these studies will provide information on the central question of how the bacteriocin molecule penetrates the outer membrane barrier to reach its apparent site of action, the cytoplasmic membrane.

In addition, we have characterized the genetic and physiological effects of the tolG mutation.

Methods Employed:

1. Purification of the TolG protein: Membranes are prepared from E. coli tol+ cells and solubilized with detergent. Protein purification has

apparently been accomplished in a single step using preparative polyacrylamide gel electrophoresis in the presence of detergent.

2. Genetic characterization of tolF mutants has been accomplished using standard techniques such as conjugation, formation of partial diploids and transduction using phage P^1.

Major Findings:

1. The purification of the TolG protein has apparently been accomplished. The purified material runs as a single band in the two different gel electrophoresis systems. {T. Chai}

2. The mutation in tolF mutants has been found to cotransduce with the pyrD locus. The tolF mutant may be phenotypically identical with the cmlB mutant, isolated resistant to chloramphenicol. Studies with F^1 episomes show that both tolF and tolG mutations are recessive.

Proposed Course of the Project:

1. We will isolate mg quanities of the TolG protein. This material will be used to determine the chemical and antigentic composition of the TolG protein. In addition, the purified material will be the starting point in an attempt to reconstitute bacteriocin sensitivity by adding purified TolG protein to E. coli tolG mutants whose outer membrane is missing this component. We will monitor the appearance of bacteriocin sensitivity among the tolerant cells using the intracellular level of ATP as a probe. This is a very sensitive assay and in bacteriocin sensitive cells, addition of bacteriocin results in the rapid loss of 90% of intracellular ATP whereas no decrease is noted where bacteriocin-tolerant cells are treated. Thus estimation intracellular ATP is a simple and rapid means whereby the reconstitution of bacteriocin sensitivity can be measured.

2. Studies with tolF mutants will be continued in an attempt to clarify the relationship of the tolF and cmlB loci. In addition, the genetic and physiologic characterization of the tolD locus, which has been positioned near cmlB, will have to be included in this work.

Keyword Descriptors:

Bacteriocin, Bacteriocin-tolerant, membrane protein, outer membrane.

Honors and Awards:

None

Publications:

None

Project No. Z01 AM 18000-10 LBM
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hormone-Dependent Differentiation of Mammary Gland.

Previous Serial Number: NIAMDD-LBM-7.

Principal Investigator: Dr. Yale J. Topper.

Other Investigators: Dr. Takami Oka, Dr. Barbara K. Vonderhaar
Dr. Colette S. Freeman, Dr. L. Zwierzchowski.

Cooperating Units: Dr. M. Wilchek, NIAMDD, Clinical Endocrinology Branch.

Man Years:

Total: 6.5
Professional: 4.5
Other: 2

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of the mammary gland. This includes attempts to understand the mechanism of action of insulin, prolactin and placental lactogen.

Methods Employed - Mammary gland explants, mammary epithelial cells or mammary cell nuclei are cultured in synthetic media. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. DNA and RNA synthesis are studied using conventional methods. DNA synthesis is inhibited by the addition of 1-β-D-Arabinosylcytosine or fluorodeoxyuridine. Insulin-Sepharose, prolactin-Sepharose and placental lactogen-Sepharose are made by conventional methods. The intracellular accumulation of α-aminoisobutyric acid (AIB) is followed with the use of ^{14}C-AIB of ^{3}H-AIB. Glucose oxidation is assessed by measuring the conversion of ^{14}C-Glucose to $^{14}CO_2$.

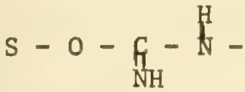
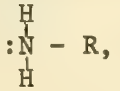
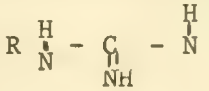
Major Findings - 1. Mammary epithelial cells from mature virgin and primiparous mice are very similar. Both are proliferatively and developmentally dormant, insulin-insensitive and non-secretory. Both can be converted into a secretory state *in vitro*. We have discovered the only known difference between these cells. Cells from the virgin animal must necessarily traverse the cell-cycle as a prerequisite to differentiation. Cells from the primiparous animal do not have this requirement. It has been concluded from these and other observations that the two cell-types are arrested in different parts of the G_1 phase of the cell cycle. This may have some bearing on the epidemiological observation that early pregnancy in humans affords significant protection against mammary cancer.

2. It is commonly held that polypeptide hormones exert their characteristic biological effects by impinging on the plasma membrane, without the necessity of entering the target cell. We have re-examined this question, using nuclear preparations from mammary cells. A characteristic effect of prolactin and placental lactogen on intact mammary cells is enhancement of RNA synthesis. Surprisingly, we have observed that these hormones accelerate RNA synthesis by the nuclear preparations. The effect is both cell and hormone specific. The relationships between such effects and the plasma-membrane theory of polypeptide hormone action will be studied.

3. We reported previously that, under conditions commonly employed with polypeptide hormone-Sepharose complexes, a form of insulin (ILM) could be solubilized from the complex, and that ILM has greater biological activity than insulin on mammary cells. This may necessitate re-evaluation of certain other studies with hormone-Sepharose preparations in which it was assumed that the hormone remained covalently linked to Sepharose during the reaction with target cells.

The production of soluble super-active polypeptide hormones in this manner is not unique to insulin. Corresponding materials prepared from prolactin-Sepharose and placental lactogen-Sepharose have also been shown to have greater biological activity on mammary cells than the native hormones.

The structure of the hormone Sepharose complexes has been shown to be


$\mathrm{S - O - \underset{\overset{\|}{NH}}{C} - \overset{H}{\overset{|}{N}}}$ - hormone, in which S = Sepharose. The release of soluble hormone-like material involves nucleophilic attack on the O - C bond by  $\mathrm{:\underset{H}{\overset{H}{N}} - R}$, generating  $\mathrm{R\overset{H}{\underset{\cdot}{N}} - \underset{\overset{\|}{NH}}{C} - \overset{H}{\overset{|}{N}}}$ - hormone, the super-active hormone.

ILM has also been shown to have biological activity greater than native insulin when tested on other target cells. Although insulin itself does not enhance the rate of glucose oxidation by diaphragm from C57Bl/6J *ob/ob* mice, ILM does stimulate the oxidation. Furthermoe adipose tissue from these obese mice is about 50 times as sensitive to ILM in terms of glucose oxidation as it is to native insulin. With corresponding tissues from lean C57Bl mice, ILM and insulin are equipotent. It is apparent that any palliative advantage of ILM would be on tissues which are resistant to native insulin. Further information about the chemical structure of ILM should contribute significantly to our understanding of the biological activity of insulin.

Proposed Course of Project:

1. Further consequences of the observed difference between mammary cells from virgin and primiparous mice will be sought.

2. We will attempt to determine whether the stimulation of isolated mammary nuclei by prolactin and placental lactogen involves the participation of any other cellular organelles present in the nuclear preparation.

3. In terms of ILM, it will be of interest to:

    (a) determine whether similar modification of insulin occurs physiologically at peripheral sites;

    (b) determine whether ILM can exert super-insulin effects on insulin-resistant tissues from other species, both in vitro and in vivo.

Significance to Bio-medical Research and the Program of the Institute:

1. The discovery that, in certain instances, hormones can exert their biological effect only when the target cell is in a particular phase of the cell-cycle offers new opportunities for the study of hormone action. Knowledge of distinctive characteristics of the cell during the responsive phase should facilitate elucidation of the mechanism of the hormone response.

2. The biological implications of the soluble super-insulin described in this report are not known. Perhaps no physiological counterpart of it exists. Alternatively, it, or an equivalent, may represent the most active form of the hormone, formed by interaction between insulin and peripheral sites in vivo. In either case, more information about its chemical structure should contribute significantly to our understanding of the biological activity of insulin.

Honors and Awards: None

Keyword Descriptors: Mammary differentiation; cell-cycle; polypeptide hormones; super-active insulin; super-active prolactin; super-active placental lactogen; insulin-resistance; hormone-Sepharose; plasma membrane; nuclei.

Publications:

Topper, Y. J. and Vonderhaar, B. K.: Concerning the role of critical-cell proliferation in differentiation of mammary epithelial cells. In Clarkson, B. and Baserga, R. (Eds.): Control of proliferation in animal cells. Cold Spring Harbor Laboratory, 1974, pp. 843-852.

Topper, Y. J., Oka, T. and Vonderhaar, B. K.: Techniques for studying development of normal mammary epithelial cells in organ culture. In Hardman, J. G. and O'Malley, B. W. (Eds.): Methods in Enzymology, New York, Academic Press, 1975, Vol. XXXIX, pp. 443-454.

Oka, T., Perry, J. W. and Topper, Y. J.: Changes in insulin-responsiveness during development of mammary epithelium. J. Cell Biol. 62: 550-556, 1974.

Oka, T. and Topper, Y. J.: A soluble super-active form of insulin. Proc. Nat. Acad. Sci. USA 71: 1630-1633, 1974.

Vonderhaar, B. K. and Topper, Y. J.: A role of the cell cycle in hormone-dependent differentiation. J. Cell Biol. 63: 707-712, 1974.

Topper, Y. J. and Oka, T.: Some aspects of mammary gland development in the mature mouse. In Larson, B. L. and Smith, V. R. (Eds.): Lactation - Comprehensive Treatise. New York, Academic Press, 1974, Vol. 1, pp. 327-348.

Chomczynski, P. and Topper, Y. J.: A direct effect of prolactin and placental lactogen on mammary epithelial nuclei. Biochem. Biophys. Res. Comm. 60: 56-63, 1974.

Vonderhaar, B. K. and Topper, Y. J.: Super-active forms of placental lactogen and prolactin. Biochem. Biophys. Res. Comm. 60: 1323-1330, 1974.

Wilchek, M., Oka, T. and Topper, Y. J.: The structure of a soluble super-active insulin is revealed by the nature of the complex between cyanogen bromide-activated Sepharose and amines. Proc. Nat. Acad. Sci. USA 72: 1055-1058, 1975.

Oka, T. and Topper, Y. J.: Insulin-unresponsive tissues respond to super-active insulin-like material. Science, in press.

Project No. Z01 AM 18001-01 LBM
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Phospholipid Stimulation of Glucose 6-Phosphate Formation in Brain.

Previous Serial Number: NIAMDD-LBM-8.

Principal Investigator: Dr. F. Eisenberg, Jr.

Other Investigators: Dr. A. L. Majumder.

Cooperating Units: None

Man Years:

Total: 2
Professional: 2
Others: 0

Project Description:

Objective - Glucose 6-phosphate, the primary fuel of brain, is an unstable metabolite, quickly disappearing upon interruption of the blood supply to that organ. Nevertheless, unsupplemented isolated brain fractions are capable of synthesizing glucose 6-phosphate by an unknown pathway which can be stimulated by phospholipids. The nature of this pathway is under investigation.

Methods - Brain homogenates and subcellular fractions, incubated in Tris buffer with various additions, were analyzed for glucose 6-phosphate by gas-liquid chromatography (GLC) of the trimethylsilyl ether by a method developed specifically for this and related studies. For the estimation of radioactivity in glucose 6-phosphate from putative precursors, the precursor and product were separated chemically by differential solvent extraction. Glucose 6-phosphate was then purified by preparative GLC and condensed from the gas stream for liquid scintillation counting.

Major Findings - Glucose 6-phosphate formation was first observed in this laboratory as a concomitant of phosphatidyl inositol breakdown in brain homogenate stimulated by deoxycholate. From this circumstance and the similarity in kinetics of formation and yield between water soluble products of phosphatidyl inositol degradation and glucose 6-phosphate, it was tentatively concluded that glucose 6-phosphate was derived from a novel phospholipid of brain similar to those recently described in bacteria. Supporting this hypothesis was the finding that whole brain lipid could replace deoxycholate in stimulating brain low-speed supernatant to produce glucose 6-phosphate. On fractionation of the lipid, however, it was found that ordinary phospholipids like phosphatidyl choline and phosphatidyl ethanolamine could replace the whole lipid. Clearly the phospholipid was not a substrate but a stimulant and the search for the substrate was directed elsewhere. Addition of various inhibitors has failed to implicate known pathways of glucose 6-phosphate formation, since none of them has inhibited formation of the ester. Glucose-^{14}C failed to incorporate radioactivity into glucose 6-phosphate. Protein-free extracts of brain added to dialyzed low-speed supernatant serve as sources of phospholipid-stimulated glucose 6-phosphate and/or of a cofactor required for the reaction.

Significance to Biomedical Research and the Program of the Institute - Phospholipids may play a dynamic role in membrane function in addition to their obvious structural role. Since changes in membranes are important in cellular and organellar function, the elucidation of underlying membrane chemistry is essential to the recognition of normal and pathological states.

Proposed Course of Project - This system will be investigated further in the hope of defining both substrate and enzyme. The precise nature of the phospholipid requirement will be studied to get some insight into the relation of phospholipid structure to protein function.

Keyword Descriptors:

Glucose 6-Phosphate; phospholipid; brain; stimulation; gas-liquid chromatography.

Honors and Awards: None.

Publications:

Eisenberg, F., Jr.: Gas chromatographic assay of iduronic and glucuronic acids as aldonic acid butaneboronates. Analytical Biochemistry 60: 181-187, 1974.

Majumder, A. L. and Eisenberg, F., Jr.: The formation of cyclic inositol 1,2-monophosphate, inositol 1-phosphate, and glucose 6-phosphate by brain preparations stimulated with deoxycholate and calcium: a gas chromatographic study. Biochem. Biophys. Res. Comm. 60: 133-139, 1974.

Eisenberg, F. Jr.: Myoinositol 1-phosphate. In Bergmeyer, H. U. (Ed.): Methods of Enzymatic Analysis ed. 2. New York, Academic Press, Inc., 1974, pp. 1337-1341.

Chen, C. H.-J., and Eisenberg, F., Jr.: Myoinosose-2 1-phosphate: An intermediate in the myoinositol 1-phosphate synthase reaction. *J. Biol. Chem.* 250: 2963-2967, 1975.

Project No. Z01 AM 18002-02 LBM
1. Biochemistry and Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Investigations on the Biochemical Abnormality Underlying Human Cystinosis.

Previous Serial Number: NIAMDD-LBM-9

Principal Investigator: Dr. Frank Tietze

Other Investigators: None

Cooperating Units: Dr. Joseph D. Schulman and Dr. Jean Butler, National Institute of Child Health and Human Development.

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives - Human cystinosis is a rare and often fatal inherited disease characterized by excessive intralysosomal storage of the amino acid cystine in certain cells of the body. The purpose of this investigation is to define the biochemical basis of the disease as a prerequisite to rational therapeutic control.

Methods Employed - Cultured skin fibroblasts from cystinotic patients exhibit characteristically high intracellular levels of cystine and therefore constitute a convenient source of living material for biochemical investigations of this disease. For the purposes of this investigation normal and cystinotic cells were incubated under varying conditions of temperature and pH and the comparative intracellular levels of free cystine determined in an amino acid analyzer. In other experiments normal and cystinotic cells were grown in the presence of 35S-labeled cystine and the protein-free extracts subsequently subjected to high voltage paper electrophoresis in formic acid; the resulting spectrum of labeled metabolites was then examined in a strip scanner.

Major Findings - The level of intracellular free cystine in cystinotic cells is markedly sensitive to small increases in incubation temperature.

For example, cells incubated at 39° for two days lose one-half to two-thirds of their cystine content as compared with control cells maintained at 37°. There was no indication of cell damage as a result of the higher temperature as determined by several parameters of cell viability. Furthermore, the effect of temperature was completely reversible: cells which had been exposed to the higher temperature for 2-4 days and subsequently returned to their former condition at 37° regained their original intracellular content of cystine.

The effect of the pH of incubation medium on intracellular cystine content of cystinotic cells was also investigated. Variable results were obtained, depending on the particular cell line selected. One cell line in particular exhibited markedly increased intracellular levels (2-4 fold) of cystine as the pH was increased from 6.8 to 8.0; this effect was also found to be reversible.

Protein-free extracts from normal and cystinotic cells exposed to 35S-cystine were examined by radioactive strip scanning following high voltage paper electrophoresis. Both cell types showed a prominent peak of radioactivity corresponding to reduced glutathione and minor peaks corresponding to oxidized glutathione and an unidentified component with positive mobility intermediate between cystine and cysteamine. Cystinotic extracts showed additionally a large peak of cystine but no other unique area of radioactivity. These results are consistent with the idea that the elevated levels of cystine in cystinotic cells are not the consequence of blockage in the catabolic pathway of cystine at a point one or more steps removed from the amino acid itself.

Significance to Biomedical Research - Among the proposals to account for the characteristic storage of cystine in lysosomes of cystinotic cells are suggestions relating to (1) impaired intra- or extra-lysosomal metabolism of the amino acid; (2) abnormal lysosomal membrane structure resulting in entrapment of an otherwise normally diffusible metabolite; (3) defective or absent carrier or transport agent essential for the egress of cystine from the lysosomal organelle. Demonstration of a membrane abnormality as the basis of cystinosis would constitute a unique example of a lysosomal storage disease arising from impaired transport across the lysosomal membrane and to this extent would add new knowledge concerning the origin of this category of disease.

Proposed Course of Project - Work will continue on the unresolved problem of the basis of cystine storage in cystinosis. Specific approaches will include the use of artificial lipid vesicles (liposomes) as carriers of chemical or enzymic reagents directly into the lysosomes of cystinotic fibroblasts and to study their effect on the metabolism or disposal of cystine therein. Hopefully this technique will also provide a method for the introduction of labeled cystine into lysosomes of normal cells so as to determine for the first time the intralysosomal metabolism, if any, of this amino acid.

Keyword Descriptors:

Cystine; cystinosis; fibroblasts; high-voltage electrophoresis; lysosomes; membranes; storage disease; tissue culture.

Honors and Awards: None

Publications: None

Project No. Z01-AM-18003-02 LBM
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Role of Spermidine in Hormone Dependent Differentiation of Mammary Epithelium *In Vitro*.

Previous Serial Number: NIAMDD-LBM-10.

Principal Investigator: Dr. Takami Oka.

Other Investigators: Dr. Kazutaka Kano, Mr. John W. Perry.

Cooperating Units: None

Man Years:

Total: 1.5
Professional: 1.5
Others: 0

Project Description:

Terminal differentiation of mouse mammary epithelium is elicited *in vitro* in the presence of insulin, glucocorticoid and prolactin. Under the influence of these hormones, nonsecretory mammary cells become secretory in appearance and synthesize a large amount of milk proteins such as casein and α-lactalbumin. Previously we have shown that the effect of glucocorticoid can be simulated by spermidine, a polyamine which accumulates extensively in mammary epithelium during lactation. In organ culture, addition of spermidine, together with insulin and prolactin, produces a marked increase in the synthesis of milk proteins, which is similar to that produced by the combination of insulin, glucocorticoid and prolactin. The involvement of spermidine in milk protein formation is further supported by the observation that the combination of these three hormones enhances the cellular concentration of spermidine before the accelerated synthesis of the milk proteins occurs in cultured mammary cells.

Based on the above findings, we have investigated the mechanism whereby the interplay of the hormones stimulates spermidine formation during mammary development *in vitro*. Emphasis is placed on the effects of hormones on the activities of several enzymes which are required for spermidine formation. The biosynthesis of spermidine involves the following steps: Arginine $\xrightarrow{(1)}$ ornithine $\xrightarrow{(2)}$ putrescine $\xrightarrow{(3)}$ spermidine.

The experimental procedures employed in this investigation include organ culture of mouse mammary gland, preparation of mammary epithelium free of fat cells, isolation and quantification of spermidine, spermine and putrescine, enzyme assay for ornithine decarboxylase, S-adenosylmethionine decarboxylase, arginase, phosphodiestrase, adenyl cyclase and lactose synthetase, and determination of the amount of cyclic nucleotides. The details of these procedures are fully described in the list of publications below.

The activity of arginase, an enzyme which catalyzes the formation of ornithine (step 1) is stimulated by prolactin in the presence of insulin. The maximal increase occurs during 2 - 3 days of incubation. Glucocorticoid by itself, or prolactin alone or the combination of the two exerts no stimulatory effect. Insulin alone maintained the initial level of the arginase activity. In addition, it was found that the conversion of arginine to spermidine is enhanced by prolactin, presumably due to stimulation of arginase activity.

When mammary explants were cultured in medium in which the concentration of arginine was reduced from 4mM to 0.2mM, the triple hormone combination produced very little increase in the milk protein synthesis. However, addition of ornithine or putrescine or spermidine to such a medium restored the increase in the milk protein synthesis. These results strongly suggest that arginine is converted, through ornithine and putrescine, to spermidine, which is required for milk protein synthesis.

The activity of ornithine decarboxylase, which catalyzes the formation of putrescine from ornithine (step 2), is controlled by several factors. First, the activity increases very rapidly by simply incubating the tissue explants in organ culture medium. The increase occurs as early as one hour after the incubation, and reaches the maximum at 3 - 4 hours. Secondly, addition of insulin augments the increase in the enzyme activity. Thirdly, prolactin, in the presence of insulin and/or glucocorticoid enhances the enzyme activity. In all cases, the increase in the enzyme activity is completely blocked by inhibition of protein synthesis, but only partially blocked by inhibition of RNA synthesis. Moreover, addition of cyclic AMP derivatives, but not cyclic GMP derivatives, causes enhancement of the enzyme activity. During the first few hours, the activities of adenyl cyclase and phosphodiestrase increase rapidly, with a net reduction of cellular cyclic AMP level. Since the effect of cyclic AMP is additive to that of insulin and prolactin, it appears that the mode of action of cyclic AMP is different from those of insulin and prolactin.

Possible involvement of cyclic AMP in regulation of ornithine decarboxylase activity is currently being examined.

S-adenosyl-L-methionine decarboxylase plays a key role in the biosynthesis of spermidine by providing decarboxylated S-adenosyl-L-methionine, which, in turn, donates a propylamine moiety to putrescine to form spermidine (step 3). It was found that glucocorticoid, together with insulin, produces a rapid and large increase in the activity of the enzyme in cultured mammary cells. These data are consistent with the view that spermidine may mediate the effect of glucocorticoid in milk protein synthesis since stimulation of enzyme activity by glucocorticoid may lead to enhanced production of spermidine.

The above observations indicate that insulin, glucocorticoid and prolactin interact to stimulate a group of enzymes that are involved in the biosynthesis of spermidine. In view of our previous observation that spermidine is necessary for milk protein synthesis, such hormonal interplay may represent a fundamental mechanism for the regulation of lactogenesis in the development of mammary gland.

The polyamine, spermidine, is present ubiquitously in living cells, and is currently considered to play an important regulatory role in the processes of growth and development. The mammary gland system described above is one of the excellent models to study the function of this polyamine in cellular development. We intend to pursue further the elucidation of the regulatory mechanism for the biosynthesis of spermidine and attempt to dissect the mechanism of action of spermidine at subcellular and molecular levels. An understanding of the function of spermidine can serve to reveal the complex regulatory mechanisms of cellular growth and development.

Honors and Awards: None

Keyword Descriptors: Mammary gland development; hormone action; spermidine; polyamine; organ culture; ornithine decarboxylase; cyclic nucleotide; arginase; glucocorticoid; S-adenosyl-L-methionine decarboxylase; insulin; prolactin.

Publications:

Oka T. and Perry, J. W.: Spermidine as a possible mediator of glucocorticoid effect on milk protein synthesis in mouse mammary epithelium *in vitro*. *J. Biol. Chem*. 249: 7647-7652, 1974.

Oka, T. and Perry, J. W.: Arginase affects lactogenesis through its influence on the biosynthesis of spermidine. *Nature* 250: 660-661, 1974.

Oka, T.: Some new aspects of the function of spermidine in animal cells (In Japanese). *Protein, Nucleic Acid, Enzyme* 20: 101, 1975.

Project No. Z01 AM 18004-02 LBM
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Lesions in the Genetic Mucopolysaccharidoses.

Previous Serial Number: NIAMDD-LBM-11

Principal Investigator: Dr. Irwin G. Leder.

Other Investigators: None.

Cooperating Units: Section on Human Biochemical Genetics, Arthritis and Rheumatism Branch, NIAMDD.

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description:

Objectives - To study the hereditary biochemical defects of lysosome dysfunction which are responsible for a group of interrelated mucopolysaccharidoses and to apply the results of such studies to clinical diagnosis and therapy.

Methods Employed - Two enzymes, α-L-iduronidase and sulfoiduronate sulfatase, are purified from human urine by ammonium sulfate fractionation, molecular sieving and affinity chromatography. The purified proteins are studied for enzymatic activity and capture by fibroblasts in tissue culture.

Major Findings - Iduronidase is presently assayed by the colorimetric determination of phenol formed from the synthetic substrate, phenyl iduronide, which is difficult to prepare and is not readily available. A sensitive assay has been devised based upon the hydrolysis of tritium labeled anhydromannitol iduronide to form radioactive anhydrommanitol which is readily separated from the anionic substrate by electrophoresis or by means of ion-exchange chromatography. The labeled substrate is prepared from heparin.

Significance to Biomedical Research and the Program of the Institute - A deficiency of iduronidase or sulfoiduronate sulfatase results in the Hurler or Hunter syndrome, respectively. The development of techniques for studying these enzymes is of importance for diagnostic purposes, and their purification may make possible their clinical application in enzyme replacement therapy. The study of the uptake of purified enzymes by cells in tissue culture will clarify the nature of the specific interaction of protein molecules with the cell membrane.

Proposed Course of Project - The development of highly sensitive and simple techniques for monitoring enzyme levels will be pursued in order to facilitate the purification and study of these enzymes and in anticipation of clinical experiments.

Highly specific affinity adsorbents bearing ligands capable of binding iduronidase and sulfoiduronate sulfatase will be prepared.

Keyword Descriptors:

Iduronidase; sulfatase; mucopolysaccharidoses; Hurler Syndrome; Hunter Syndrome; lysozymes; affinity chromatography; genetic diseases.

Honors and Awards: None

Publications:

Lim, T. W., Leder, I. G., Bach, G. and Neufeld, E.: An assay for iduronate sulfatase (Hunter corrective factor). Carbohydrate Res. 37: 103, 1974.

Leder, I. G.: Thiamine, biosynthesis and function. In D. M. Greenberg, (Ed.) Metabolism of Sulfur Compounds, New York, Academic Press (in press).

Project No. Z01 AM 18005-02 LBM
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Differentiation of Lymphoid Cells.

Previous Serial Number: NIAMDD-LBM-12

Principal Investigator: Dr. Milton Kern

Other Investigators: Dr. Nobukata Shinohara

Cooperating Units: None

Man Years:

Total: 3
Professional: 2
Others: 1

Project Description:

Objectives - The purpose of this project is to analyze the cellular events which are prerequisite for the induction of the synthesis and secretion of immunoglobulins; with particular emphasis on the conversion of precursors of immunoglobulin-producers (B cells) and on the role of cell-free mediators derived from T cells (thymus-derived lymphocytes) in cellular cooperation.

Methods Employed - Rabbit lymphoid cells were incubated in tissue culture medium without added antigen. At 72 hours, cells were harvested, washed and incubated in leucine-free medium supplemented with [H^3]-leucine. The amount of synthesized immunoglobulin in the supernatant was assessed by specific coprecipitation using purified rabbit immunoglobulin (RIg) and antiserum against RIg. Thymocyte culture filtrate was obtained by incubating thymocytes in culture medium for 72 hours. Thymocyte extract was prepared by lysing thymocytes by ultrasonication.

Major Findings - Rabbit spleen cells when cultured *in vitro* for 72 hours undergo differentiation into IgM-producing cells in the absence of added antigen. This spontaneous IgM production requires the participation of T cells in the sense that the treatment of spleen cells with antithymocyte serum (ATS) inhibits the response, and addition of thymocytes restores it. Furthermore, addition of thymocytes to untreated spleen cells causes enhancement of IgM production.

Thymocyte culture filtrate (Fil) and thymocyte extract (Ext) were found to possess an enhancing activity on IgM production of spleen cells. In the supernatant of thymocyte culture, this activity first appears after 24 hours of incubation, and reaches its maximum at 72 hours. The activities of Fil and Ext were not additive nor synergistic when they were added into the same culture bottle at the concentration which gave the maximal stimulation. This finding suggests that the enhancing factors in the two preparations are acting at the same site and are probably identical.

Neither Fil nor Ext could restore the IgM production inhibited by the ATS-treatment of spleen cells. The magnitude of IgM production of ATS-treated spleen cells maximally stimulated by Fil or Ext was far smaller than that of untreated cells, while addition of live thymocyte completely restored and even enhanced it. This result indicates that Fil and Ext cannot completely substitute for live T cells and their function represents only a part of the multiple function of T cells as a "helper" of immunoglobulin production by B cells.

Incubation of spleen cells with Fil or Ext for a short period during the course of tissue culture revealed that the cells are insensitive to the enhancing stimulation during the first 24 hours and that most cells are sensitive between 36 to 60 hours. It was also shown that DNA synthesis precedes this stage. Furthermore, Fil and Ext were shown to have no mitogenic activities. They could exert their enhancing effect even when added together with mitotic inhibitor (hydroxyurea) at 48 hours, if DNA synthesis was allowed before the addition. These findings suggest that the target cells which are most susceptible to stimulation are those which have already experienced cell division, and enhancement by Fil and Ext does not involve further cell division.

Significance to Biomedical Research and the Program of the Institute - Elucidation of the regulatory mechanism in the induction of immunoglobulin production is of vital importance for understanding both the defence mechanism of the body and the nature of several diseases in which regulatory disorders of the immune system appear to be involved. Examples of these diseases are immuno-deficiency disease, allergic disease, auto-immune disease and neoplastic disease of lymphoid cells.

It is well established that cooperation of two types of lymphocytes, i.e. T cells and B cells, is required for the induction of immunoglobulin formation. However, the precise cellular mechanism and molecular basis for this interaction between cells are yet to be delineated. As described above, a system which we have developed enables us to study some aspects of the control mechanism of immunoglobulin production in a well-defined manner.

Proposed Course of Project - Efforts will be directed towards characterization of the T cell-derived mediator as well as assessing the significance of the mediator in specific antibody responses.

Keyword Descriptors:

Antigen-independent induction of immunoglobulin synthesis; cell cooperation; T cell mediator; non-mitogenic enhancement; target site for enhancing effects; differentiation of B cell; IgM-producing cell.

Honors and Awards: None.

Publications:

Kern, M.: Surface Immunoglobulins: Characteristics, Mobility and Role in Immune Phenomena. In Jamieson, G. A. and Greenwalt, P. J. (Eds.): Mammalian Cell Membranes. London, Butterworth & Co. (in press).

Zimmerman, D. H. and Kern, M: Differentiation of lymphoid cells: the in vitro emergence of immunoglobulin M-secreting cells. J. Immunol. 111: 761-769, 1973.

Zimmerman, D. H. and Kern, M: Differentiation of lymphoid cells: effect of serum and other mitogenic agents on the selective induction of immunoglobulin M secreting lymph node cells in tissue culture. J. Immunol.111: 1326-1333, 1973.

Okumura K., and Kern, M.: Differentiation of lymphoid cells: suppression of the induction of immunoglobulin M secreting rabbit lymph node cells by cells present in bone marrow. Cellular Immunology (in press).

Zimmerman, D. H., Okumura, K., Rabkin, C. and Kern, M.: Differentiation of lymphoid cells: the use of specific antisera to characterize the cells required for the induction of immunoglobulin M production in vitro. J. Immunol. 113: 1891-1896, 1974.

Okumura, K., Shinohara, N. and Kern, M.: Differentiation of lymphoid cells: enhancement of the induction of immunoglobulin production by thymus cells and a product secreted by thymus cells. J. Immunol. 113: 2027-2034, 1974.

Project No. Z01 AM 18006-04 LBM
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Particulate Enzymes of Carbohydrate Metabolism.

Previous Serial Number: NIAMDD-LBM-13

Principal Investigator: Dr. Marjorie R. Stetten

Other Investigators: Mr. Paul K. Goldsmith

Cooperating Units: None

Man Years:

Total: 2
Professional: 2
Others: 0

Project Description:

The objective of the project has been to study the occurence, properties and functions of some of the membrane-bound enzymes of liver and kidney which catalyze the synthesis, hydrolysis and transport of a variety of phosphorylated sugars and sugar alcohols. Of particular interest is the microsomal enzyme glucose-6-phosphatase which, in addition to its well-known hydrolytic function, has been found to be capable of catalyzing synthetic reactions utilizing such high energy compounds as inorganic pyrophosphate and carbamyl phosphate. The quantitative levels of these membrane-bound enzymes have been found to be particularly susceptible in vivo to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, cortisone and insulin administration.

We are currently studying the development of glucose-6-phosphatase and its related enzyme activities in the endoplasmic reticulum of the livers and kidneys of young rats in the hope of contributing to an understanding of the mechanism of the synthesis of biological membranes. Glucose-6-phosphatase is known to make its appearance at birth and to undergo a dramatic increase in relative specific activity during the first four days of life, followed by a gradual decrease to adult levels of activity. The enzyme has been shown to have a significant component of "latent" enzyme activity, which is apparent only after pretreatment of the membranes with detergents or controlled alkaline conditions. In contrast to earlier studies, we are exploring the enzyme levels of both rough and smooth subfractions of the membranes, as a function of the age of the animals, before and after the enzymes have been fully activated *in vitro*. So far we have found that the postnatal "overshoot" in enzyme activity observed in unfractionated microsomes is largely a property of the rough membrane fraction. The enzyme in the smooth subfraction increases more gradually with increasing age. The phenomenon of latency has been found to be quantitatively more significant in very young than in older animals. We intend to extend these developmental studies to mature and senile animals and to include studies of other enzymes involved in carbohydrate metabolism and in the synthesis of the phospholipid components of the membranes.

In the course of studies started last summer at the Marine Biological Laboratory, Woods Hole, Massachusetts, comparing particulate enzymes of the hepatopancreas of marine invertebrates with those of mammalian liver, we found that the hepatopancreas of Limulus (Xiphosura polyphemus) has an enzyme complex with many properties very similar to those of the liver glucose-6-phosphatase of rat, man and other high vertebrates. The hydrolytic and phosphotransferase activities also have an organ distribution comparable to vertebrates, since they occurred not only in the "liver" but also in the intestinal mucosa and the coxal gland, thought to correspond to the kidney of higher forms. In the organs of other invertebrate animals studied, the comparable enzyme activities either had different properties or were entirely absent. Limulus is a species which has in the past few years attracted the attention of the medical profession because of the increasing use of a preparation made from its circulating blood cells in a very sensitive test for bacterial endotoxins. Since surprisingly little appears to be known about the general biochemistry of Limulus, we expect to extend our studies to other aspects of carbohydrate metabolism in this interesting animal.

Keyword Descriptors:

Carbohydrate metabolism; glucose-6-phosphatase; microsomal membranes; phosphotransferase; limulus hepatopancreas; development of membrane enzymes; rough and smooth microsomes; enzyme latency.

Honors and Awards: None.

Publications: None.

ANNUAL REPORT OF THE LABORATORY OF CHEMISTRY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry of Fluoroimidazoles

Our discovery of a photochemical route to ring-fluorinated imidazoles resulted in the syntheses, in the first phase of the program, of 2- and 4-fluoroimidazoles, the corresponding histamines, and histidines. The widespread biochemical and pharmacological activities of these (and related) compounds, as well as their wholly unpredicted chemistry, propelled the initiation of the second phase - preparation and study of a series of more complex fluoroimidazoles, particularly the difluoro compounds. The first of these, 4,5-difluoroimidazole, has now been prepared and, true to its heritage, has opened a new box of chemical and biochemical surprises. In the course of trying to explain why 2-fluoroimidazoles are chemically reactive toward nucleophiles (while 4-fluoroimidazoles are totally inert), we find that 4,5-difluoroimidazoles are also reactive. In the course of trying to explain why 2-fluoroimidazoles undergo facile isotope exchange at H-4, we find that 4-fluoroimidazoles fail to exchange at H-2; furthermore, fluorine seems to be the only substituent at C-2 which permits facile exchange at H-4. Yet, these anomalies in chemical behavior are welcome findings, since each has been used to advantage in some biochemical application, as described below. (Y. Takeuchi, K. L. Kirk, L. A. Cohen).

Cyclic AMP and GMP Phosphodiesterases

The demonstration that imidazole activates the AMP enzyme about 1.5-fold (presumably in a secondary, regulatory site) prompted our exploration of other imidazoles. While 2- or 4-fluoroimidazole proved to have no significant activity, 4,5-difluoroimidazole inhibits the enzyme to a modest degree, and 4-fluoroimidazole-5-carboxylic ester is even more effective. Compounds for inhibition of the enzyme are vital to study of the mechanisms of action of cyclic AMP and for identification of the various hormones which stimulate its release. The clinical possibilities associated with regulation of the cellular levels of cyclic AMP and GMP are also considerable. Some of the inhibitors currently in use (caffeine, theophylline) are limited in value because of their poor specificities, high levels required, and their own pharmacological activities. The fluoroimidazoles are competitive inhibitors despite their lack of basicity, but this very property may be important in enhancing specifity and reducing side effects. (K. L. Kirk, R. Harshman, L. A. Cohen)

Antiviral and Antinucleotide Activities of Fluoroimidazoles

Ribosides of several fluoroimidazoles have been synthesized and are being tested, along with the fluoroimidazoles themselves, as antiviral agents. To date, a riboside of 4-fluoroimidazole-5-carboxamide has been found to parallel the specificity and activity of ribavirin (virazole), a triazole derivative just entering clinical testing. In both cases, activity is not achieved by stimulation of interferon production, but possibly by competitive inhibition of IMP dehydrogenase, an enzyme vital to polynucleotide biosynthesis. In fact, this fluorinated riboside has been shown to be equal or better than ribavirin in blocking DNA and RNA synthesis in cell cultures. (E. De Clercq, M. Luczak, J. C. Reepmeyer, K. L. Kirk, and L. A. Cohen)

Histidine Ammonia-Lyase

Our earlier work had shown 4-fluorohistidine to be the only effective competitive inhibitor for histidine with this enzyme. While the catabolism of histidine has been elucidated, very little is known about the biological role of the enzyme or about the histidine catabolites, except that an inherited lack of the enzyme is fatal (histidinemia). Other inhibitory analogs of histidine have now been found: 2-fluorohistidine, 2-aminohistidine, 4-nitrohistidine. Analysis of kinetic data, reversibility, isotope incorporation, and isotope effects prove conclusively that the slow step for this enzyme is not the breakdown of the aminoenzyme, as previously supposed, but the almost concerted loss of a β-hydrogen and the bound amino group. On further study with 4-nitrohistidine, both the metal requirement and the isotope effect are eliminated. It is thought that the rate-determining step has now been advanced to that of formation of the enzyme-substrate complex. This possibility of fixing the rate-determining step of an enzymatic process at one of three different points in the sequence by small variations in the substrate is unique for enzymology and should prove the basis for new general concepts in the design of inhibitors. (C. Klee, P. McPhie, K. L. Kirk, and L. A. Cohen)

Ribonuclease

Ribonuclease-S-peptide has been synthesized with 4-fluorohistidine in place of histidine-12. While the analog combines very readily with ribonuclease-S-protein, the adduct shows no enzymatic activity. On the basis of numerous physicochemical and biochemical criteria, the adduct appears to have a conformation very close to that of ribonuclease-S. Most impressive is the fact that substrate analogs protect this adduct against proteolytic digestion to the same degree as ribonuclease-S. It is concluded, therefore, that the fluoroimidazole ring, with a pK 4-5 units lower than that of imidazole-12, is unable to function as a general base in the catalytic process of the enzyme without interfering with binding. This observation provides the most conclusive support, to date, for the role assigned to histidine-12 in ribonuclease. Parallel studies with 2-fluorohistidine, and with fluoro analogs at other sites along the chain, are in progress. (B. M. Dunn, C. DiBello, K. L. Kirk, L. A. Cohen, and I. M. Chaiken)

Urocanase

The product of deamination of histidine by histidine ammonia-lyase, urocanic acid, is converted further by urocanase into a derivative of glutamic acid. This pathway serves as a source of one-carbon units for other biosynthetic schemes. Whether histidine supplies a carbon unit for specific pathways or for a general pool, and whether it can be replaced by alternative one-carbon sources, has yet to be determined. While the role, if any, of urocanic acid is unknown, it is thought significant that high concentrations of the substance are found in mammalian skin, and disappears when that skin becomes cancerous. The product of deamination of 2-fluorohistidine, 2-fluorourocanic acid, has been found an extremely potent inhibitor of urocanase. We hope to use this inhibitor as a tool for establishing the significance of the overall degradative pathway in mammalian biosynthesis, and for the role of urocanic acid in mammalian skin. (C. Klee, K. L. Kirk, and L. A. Cohen)

Florohistamines as Selective Histaminergic Agonists

Two types of receptor, termed H1 and H2, have been identified as mediators of histamine's biological actions. Characterization of these receptors, and of histamine's biological roles, have been hampered by lack of agents which selectively bind one type. Recently, it has been reported that various substitutions at the 2 or 4-carbon of histamine's ring selectively decrease its binding for H2 or H1 receptors, respectively. Thus, 2-phenylhistamine retains some 20% of histamine's potency at H1 receptors, but is less than 0.1% as effective at H2 receptors. Our preliminary studies suggest that 2-fluorohistamine has equal or greater affinity for H1 receptors (in guinea pig ileum) than the most effective H1 agonist, 2-aminoethylthiazole. We are preparing to examine its specificity in several histamine receptor systems. By analogy, 4-fluorohistamine might act as a selective agonist for H2 receptors. 2-Fluorohistamine has also been found an effective stimulator of cAMP formation in brain slices, which contain both H1 and H2 receptors. We are currently studying its specificity in this system by use of selective histamine antagonists. (R. K. Dismukes, J. Daly, K. L. Kirk, and L. A. Cohen)

Hormone-Releasing Factors

Several small and simple peptides have been found to be powerful triggers for the release, or agonists for the biosynthesis, of certain hormones. Luteinizing hormone-releasing factor (LHRF, a decapeptide) and thyrotropic hormone-releasing factor (THRF, a tripeptide) have been studied in detail; each sequence contains a single histidine residue which has been pinpointed as critical to binding or activity. These sequences, with 4-fluorohistidine as a replacement for histidine, are inactive; however, the 2-fluorohistidine analogs show 20-30% activity. The results are puzzling, since a drop of 4-5 pK units in the imidazole ring should remove binding ability. Such analogs offer a powerful tool for elucidation of essential binding factors and the trigger mechanisms. Furthermore, the chemical reactivity of the 2-fluoro substituent opens possibilities for covalent affinity labelling of the receptor sites. (M. Monahan, K. L. Kirk, and L. A. Cohen)

Fluorohistidines in Protein Biosynthesis

Our observation of exchange of H-4 in 2-fluorohistidine opened the way for a facile preparation of 4-tritio-2-fluorohistidine by simple exposure to T_2O at pH 10-11. When the labelled amino acid was administered to mice subcutaneously, it entered the main blood pool immediately. After 10 min, various organs (including brain) showed tritium levels 2-7 times that of the blood. After 72 hr, two-thirds of the tritium count was still unexcreted, and half of this amount was found in insoluble protein fractions. Since we had demonstrated that replacement of the 2-F group by any other substituent prevents exchange at H-4, and since all the tritium could be back-exchanged from the protein fractions at pH 11, the analog must have entered into protein synthesis in place of histidine (i.e., the 2-fluoroimidazole moiety was intact). In support of this thesis, it could be shown that actinomycin blocked incorporation of the labelled amino acid into liver ribosomes _in_ _vitro_, and cycloheximide blocked incorporation in the whole mouse. In further studies, 2-fluorohistidine was found to inhibit the isoproterenol-stimulated synthesis of N-acetyl transferase in cultured male rat pineal gland and to block incorporation into the enzyme of ^{14}C-histidine but not of ^{14}C-leucine. The tritiated 2-fluorohistidine is incorporated into new glandular protein, and this process is inhibited by histidine. The possibility is being explored that fluorohistidine replaces histidine at the active site of one (or more) enzymes in the system. (D. Klein, K. L. Kirk, C. R. Creveling, L. A. Cohen)

Antibiotic Properties of Fluoroheterocycles

Concentrations as little as 10^{-5} M of 2-fluorohistidine cause an inhibition of cell growth of E. coli (wild) which is essentially complete in 3 hr at 37° and which is reversible by histidine (20 μg/ml). During the course of the inhibition, the mass of the culture increases about threefold but the number of viable cells increases about ninefold. These data suggest that the bacteria, which contain two to three copies of their chromosome under the conditions of growth prior to administration of the inhibitor, are unable to carry out further chromosomal replication in the presence of the drug but are able to undergo cellular division. Cells grown with tritiated 2-fluorohistidine incorporate radioactivity into material insoluble in cold trichloroacetic acid. About 50% of the counts seem to be incorporated into protein as 2-fluorohistidine. Preliminary evidence suggests that the remaining counts may be present in a bound metabolite of 2-fluorohistidine. (T. Furano, K. L. Kirk, R. Harshman, L. A. Cohen)

Essentially total inhibition of growth is also observed with 2-aminohistidine, while 4-fluorohistidine and 2-fluorothiazoles retard but do not inhibit growth. (T. Furano and K. L. Kirk)

2-Aminohistidine as a Analog of Arginine

2-Aminohistidine is recognized by trypsin as a cyclic analog of arginine, since peptides of the former amino acid are hydrolyzed by the enzyme. We are now exploring the possibility of converting histidine into 2-aminohistidine within the peptide sequence, as a device for creating an additional site for tryptic cleavage. (K. L. Kirk and L. A. Cohen)

^{18}Fluoroimidazoles as Radiotracers

^{18}Fluoro analogs of biological materials are of interest and potential use as internal scintigraphic agents. An animal or human organ containing such a compound may be scanned by a γ-radiation detector or film, providing a record of exceptional clarity and resolution. Despite the fact that the radiation is powerful enough to pass through the human body, the very short half-life of ^{18}F (30 min) exposes the individual to an insignificant total radiation dose. Our findings that 2-fluorohistidine passes the blood-brain barrier very rapidly suggests that the radioactive amino acid may be useful in radiodetection of brain tumors, which are notoriously difficult to detect. Further, our observation that the fluorine of 2-fluorohistidine is subject to nucleophilic displacement suggests that labelling may be achieved by simple and rapid exchange with ^{18}fluoride ion, as obtained directly from the pile. This possibility is being explored in the radiochemical laboratory of the Roumanian Atomic Energy Commission, Bucharest. (A. I. Balaban, C. Mantescu, K. L. Kirk, and L. A. Cohen)

Heteroannular Imidazoles

The chemical reactivity of 2-fluoroimidazoles led to the accidental observation that such compounds can undergo facile trimerization to cyclic polyimidazoles, with loss of the fluorine atoms. The products appear to be heteroannular aromatic systems, since they have ultraviolet spectra characteristic of polynuclear aromatics (imidazoles themselves have no significant absorption) and have pK values below 1 or 2 (imidazole pK = 7). Efforts are in progress to obtain the cyclic tetramers, which would be imidazole analogs of porphyrins. (Y. Takeuchi, K. L. Kirk, L. A. Cohen)

Tritium-Labelled Peptides

Our studies on isotope exchange in the fluoroimidazoles led us to recognize the utility of similar exchange in the imidazole ring of histidine-containing peptides and proteins. At pH 7-9, peptides undergo slow exchange of their histidine H-2 atoms; after back-exchange of the more labile tritiums (OT, NT), the products are sufficiently stable to serve as tritium-labelled hormones for binding studies and assay purposes. (C. Klee, K. L. Kirk, L. A. Cohen)

Fluorocatecholamines

The photochemical method for introduction of fluorine has been extended to phenolic rings, and proved successful in providing the first synthesis of ring-fluorinated tyramines and catecholamines. Several collaborative projects have been initiated to study these compounds as agonists or antagonists of the natural compounds in various metabolic and neural systems. (K. L. Kirk)

Labelling by Halogen Reduction

An alternative method of labelling with deuterium or tritium involves hydrogenolysis of carbon-halogen bonds in an atmosphere of the hydrogen isotope. While this method has been used for a number of aromatic systems, there exists the danger of transition metal-catalyzed scrambling, particularly in slow reactions. Furthermore, this method is not applicable to peptides or proteins containing carbon-sulfur bonds, which either poison the catalyst or are themselves hydrogenolyzed. We have found that zinc-copper couple, in aqueous media (pH 3-5) at 25°, is extremely effective in removing the halogen from iodo- or bromoaromatics without scrambling, and are exploring the use of this method for labelling of peptide hormones by reduction of the iodinated analogs. Rather than a true reduction, we believe the reaction to involve hydrolysis of an intermediate arylzinc halide. (W. Nagai, K. L. Kirk, L. A. Cohen)

Labelling of Biological Materials with Radioiodine

Radioiodination of microquantities of peptides hormones, for the purposes of radioimmune assay and receptor-binding studies, often destroys all or part of the activity of the substrate because of the violence of the reaction with iodine monochloride (generated *in situ* from I^- and chloramine T). We have shown that a milder, but useful radioiodination can be achieved by generation of I_2, extraction into an organic solvent, and reaction with the substrate in a two-phase system. An even better technique involves reaction of the substrate with radioactive, insoluble polyiodosuccinimide. A water-soluble version of this polymer has been found effective in selectively iodinating highly exposed tyrosine residues of membrane proteins. (R. Jerussi and L. A. Cohen)

Polymer-bound Drugs and Inhibitors

The initial aim of this work was to develop a method for covalent attachment of drugs and inhibitors to a water-soluble polymer, the latter being non-antigenic, compatible with blood, and resistant to enzymatic degradation. Reaction of tyramines or catecholamines with ethylene-maleic anhydride copolymer produces a water-soluble polymer with the amine bound covalently. However, the drug is released very slowly at neutral pH, the rate of release increasing with reduction in pH, due to catalysis by adjacent carboxyl groups. While this result was unanticipated, it may prove very useful in the development of slow release forms of drugs, as well as systems in which release of a drug is triggered by an undesirable drop in local pH. More rapid release of the drug can be achieved by use of the stereopopulation control feature of backbone-alkylated analogs of the polymer. (P. A. Cohen and L. A. Cohen)

Stereopopulation Control and Models for Enzyme Catalysis

We have argued that the deformation of a substrate and consequent activation, in the course of binding to an enzyme, may contribute far more to the overall rate enhancement phenomenon (enzyme catalysis) than has previously been supposed. In an effort to support the theory, we have synthesized and studied an extensive series of intramolecular systems in which the reacting groups are frozen into van der Waals proximity and optimal orientation by appropriate substitution. In the past year, studies have continued on the kinetics and mechanism of cyclization of alkylated homophthalic acids. Four important developments have emerged from this study: (1) A curved correlation is found between log k for cyclization and the pK of the general acid catalyst, covering the range from acetic to perchloric acid, a spread of 12 pK units; the curvature was resolved into two linear correlations, representing distinct mechanisms for cyclization at the extremes of the pK region, and overlapping in the center. (2) Catalysis by general base has also been demonstrated; such catalysis is possible only if breakdown of a tetrahedral intermediate is rate-limiting. Only through a rate-enhancement device such as stereopopulation control could general catalysis of so difficult a process as anhydride formation be demonstrated. (3) Removal of one methyl groups from the trialkyl lock reduces the rate enhancement facter only 50%. This observation eliminates once and for all any arguments that the super rate enhancements observed in our studies are due to relief of steric compression; in the last case mentioned, steric compression cannot exist. (4) It now appears that stereopopulation control is indeed achieved by freezing of rotational isomerism and attendant phenomena, and that it does provide the best model for the enzyme-substrate complex available to date. (P. S. Hillery and L. A. Cohen)

We have continued our efforts to synthesize a series of imidazolealkanoic acids with conformationally locked side chains. These efforts had been hampered by a series of remarkable rearrangements, some involving dearomatization of the imidazole ring, but the desired compounds have finally been obtained and kinetic studies are in progress. The very large rate enhancement effects in this and the diacid series above demonstrate that conformational freezing can, indeed, be utilized to drive the formation of high energy bonds such as anhydride and acylimidazole. (J. Reepmeyer and L. A. Cohen)

A series of dihalogenated 6-hydroxyhydrocoumarins has been prepared in order to study the effect of halogen size on rate enhancement by conformational restriction. (M. King and L. A. Cohen)

Studies are continuing on the effect of frozen charges on pK. Conformationally locked imidazolealkanoic acids and amines are being examined as models for the imidazole ring in proteins, and for the perturbation of pK by neighboring charged groups. (H. Yeh, J. S. Cohen, Y. Kikugawa, J. Reepmeyer, L. A. Cohen). The possibility of using the very sensitive ^{19}F magnetic resonance signal as a probe of solution conformation in peptides and in simpler analogs is being explored (H. Yeh, J. S. Cohen, K. L. Kirk, and L. A. Cohen)

Miscellaneous

N-p-hydroxyphenylmaleimide has been synthesized and iodinated. This material will be selectively coupled to immunoglobulins to provide a basis for endoradiotherapy. (P. A. Cohen and L. A. Cohen)

A series of 2,6-di-t-butyl and 2,6-dimethyl-4-substituted anisoles have been prepared in an attempt to prove the existence of a 'restoring force' in aromatic systems. These compounds will be examined by UV, ^{1}H NMR, ^{13}C NMR, and crystallography in order to test the concept. (P. A. Cohen and H. Yeh)

SECTION ON CARBOHYDRATES

Glycosidases

Various 2-acetamido-2,3-dideosey-D-hex-2-enolactones were tested for inhibitory activity against 2-acetamido-2-deoxey-β-D-glucosidase (from bull epididymis). The lactones showed high specificity for this enzyme only, and not for other glycosidases. Using gluconolactone coupled to sephacose via a benzidine linkage, it was possible to prepare an affinity chromatography column for the above glucosidase, and the enzyme was isolated in a highly pure state from bull epididymis (Pokorny and Zissis).

Anticarbohydrate Immunoglobulin

In an attempt to prepare an affinity column for immunoglobulins with anti-levan activity, *p*-aminophenyl-β-*D*-arabinofuranoside was prepared (this compound shows the saccharide half of the molecule which is identical to nor-1-hydroxymethylfructofuranoside) and coupled to bovine serum albumin. Unfortunately the resulting material did not bind to the antifructans (Zissis). Consequently, both levan (from aerobacter levanicum) and inulin were derivatized to their carboxymethyl derivatives and that was coupled to ethylene diamine. The resulting polysaccharide derivatives were then coupled to cyanogenbromide treated sephacose. The obtained product proved to be a satisfactory affinity chromatography material (Glaudemans).

To distinquish between anti-levan with β(2→6) specificity efforts are underway to isolate and purify a polysaccharide from grass which is supposedly a β(2→6) linked levan (Tomasic).

In an effort to establish if the antigalactan-immunoglobulins are really specific for β(1→6) linked D-galactosyl units, the disaccharide galactopyranosyl β(1→4) galactose was synthesized. Evaluation of its binding with antigalactans is in progress (Glaudemans and Manjula).

Hybrid recombinants of antigalactan immunoglobulins have been prepared. The following systems were studied: X-24 with T601, and X-24 with S539. he resulting recombinants ($H^{24}L^{601}$, $H^{601}L^{24}$, $H^{24}L^{539}$ and $H^{539}L^{24}$) all showed that the idiotype of an immunoglobulin is mostly associated with the heavy chain. Also it was found that the binding constants between hydrids and galactose containing ligands were very similar to those of the naturally occurring immunoglobulins (Manjula).

Finally, the binding studies between galactose-ligands and antigalactans were continued. Partially blocked galactodiose (1,2,3,4, isopropylidene derivative) as well as the 6-O-acetyl derivative of methyl β-D-galactoside bound to immunoglobulin J539 with binding constants about equal to those of their unblocked equivalent. This shows that the combining area must really be on the surface of the molecule.

Chemistry of Ketoses

For the purpose of synthesizing β-D-fructofuranosides (*cis* relationship at C-2 and C-3), fructofuranose derivatives with a non-participating group at the C-3 position and a participating group at the C-1 are desired. Several approaches are bing pursued with 1,4,6-tri-O-benzoyl-3-O-benzyl-D-fructofuranose having been obtained by one of them.

SECTION ON MEDICINAL CHEMISTRY

Cannabinoid Research

(-)-9-*nor*-9α-Hydroxyhexahydrocannabinol (9-*nor*-9α-HHC) is essentially inactive in the hot-plate test for analgesia unlike its epimer, (-)-9-*nor*-9β-HHC) which is of the order of potency of morphine in the hot-plate, Nilsen, writhing and bradykinin tests. Both epimers displayed CNS-depressant, hypotensive and anticonvulsant activity in animal tests. Four (new) similar synthetic compounds (with the 9-hydroxy group) show CNS effects but have little analgesic activity. The (-)-9-*nor*-9β-HHC, at doses which produce CNS effects, will not substitute for morphine in dependent monkeys. These compounds, none of which contain nitrogen, represent a class of potentially potent, non-addicting, analgesics, hypotensives, anticonvulsants and CNS depressants.

An improved synthesis of 9-*nor*-Δ^9-tetrahydrocannabinol (9-*nor*-Δ^9-THC) was devised. It, like the Δ^8-isomer, was similar, quantitatively, and qualitatively to Δ^8 and Δ^9-THC (the active principles of marihuana) indicating that metabolism to the 11-hydroxy congeners is not necessary for these biological activities as previously believed. However, examination of Δ^8 and Δ^9-THC and a large number of hydroxylated metabolites showed that only 11-hydroxy-Δ^8- and Δ^9-THC's exhibit strong *analgesic* activity and suggests that these major metabolites are probably responsible for this pharmacologic action. Such studies are useful in determining how the cannabinoids exert their effects and in defining structure-activity relationships (R. S. Wilson).

3-Benzazocines. Chemical and Biological Studies

(1) 8-Hydroxy-6-methyl-3-alkyl derivatives. 3-Alkyl- (C_1 to C_7) 8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocines (bicyclic) less rigid homologs of the 6,7-benzomorphans were devoid of narcotic antagonist properties and displayed low-order analgesic activity (C_1 and C_7 were about half as potent as codeine). They did give a significant correlation of analgesic activity and binding affinities to brain homogenates providing additional evidence that these binding sites are involved in centrally mediated analgesia (M. E. Rogers with W. Klee, NIMH).

(2) 2,6-Methano-hexahydro-3-benzazocines (*non*-quaternary carbon 6,7-benzomorphans). 2,9α-Dimethyl-2'-hydroxy-6,7-benzomorphan (I), not obtainable by the 12-step sequence (see 1973-1974 report) used for the β-isomer (II) has now been prepared by intramolecular cyclization (aminomercuration with mercuric acetate) of 1,2-dihydro-7-methoxy-1-(2-methylaminoethyl)-2-methylnaphthalene *via* *trans*-1,2-dihydro-4-(2-dimethylaminoethyl)-6-methoxy-3-methyl-1(2H)-naphthalenone. Also obtained was a small amount of II and 2,9α-dimethyl-8α-hydroxy-2'-methoxy-6,7-benzomorphan. Compound I, without a quaternary carbon, is as potent as pethidine as an analgesic, is without physical dependence capacity in monkeys and is a long-lasting narcotic antagonist. II was four times more potent (morphine-like in potency) and also precipitates withdrawal symptoms in morphine-dependent monkeys. It has no physical-dependence capacity. The deoxy congener of II has been synthesized

in low yield from phenylalanine via 2-benzylmethylamino-6-methoxy-3-methyl-1-phenyl-3-hexanol (H. Inoue).

(3) 2,6-Methano-11α and β-propyl-hexahydro-3-benzazocines. One of the few missing links in the 6,7-benzomorphan series was the 2,5-dimethyl-2'-hydroxy-9-propyl compound. Both the α and β isomers have been prepared in the usual way from 4-methyl-3-propylpyridine (for which a new synthesis had to be devised) and resolved into optical antipodes. The α-isomer, of about the potency of morphine, gave an ED_{50} which is in good agreement with the Hansch-calculated value. The two-times more potent β-isomer was four times weaker than the calculated value. As expected all activity resides in the levo-isomers. Many N-substituted (C_1-C_8 alkyl and allyl and cyclopropylmethyl) compounds in the α-series have been prepared from the >NH derivative obtained by a vastly improved N-demethylation procedure (K. Rice and A. E. Jacobson).

Morphine Derivatives

As β-chloromorphide was recently identified as a constituent of an illicit heroin sample in a U. S. Customs laboratory, the structure (not hitherto known with certainty), analgesic activity and acute toxicity of α- and β-chloromorphides were determined along with those of some bromo and iodomorphides. Further, these materials are reactive allylid halides and could be opiate-receptor, alkylating agents. However, binding studies showed that they are not irreversible binders (A. E. Jacobson and R. S. Wilson).

Norheroin and 6-acetylmorphine have been synthesized for the first time and found to have, as expected, low-order analgesic activity. Nevertheless, in vitro binding studies (guinea-pig ileum, brain homogenates) are being performed to further probe the nature of the narcotic receptor (K. Rice and A. E. Jacobson).

An Improved N-Demethylation Procedure

By cleavage of phenylcarbamate derivatives with 80% hydrazine, 85-95% overall yields of normorphine, norcodeine and normetazocine were obtained from the corresponding N-methyl compounds. Older methods gave, at best, 50-60% yield by more tedious processes. For norheroin and 6-acetylnormorphine which were never before prepared, special protecting groups were necessary; yields from morphine were 75-80% (K. Rice).

Biochemistry and Pharmacology

(1) Effect of opiates on PGE_1 stimulation of adenyl cyclase. A series of opiate agonists and antagonists did not affect the PGE_1-stimulation of adenyl cyclase (in homogenates or slices of guinea-pig ileum) in contrast to a published report describing morphine inhibition of the PGE_1 stimulation in rat-brain homogenates. This suggests that morphine acts by different mechanisms in the brain and ileum or that its action is not due to inhibition of PGE_1-stimulated adenyl cyclase (R. S. Wilson with W. Klee, NIMH).

(2) Inhibition of uptake of norepinephrine (NE). cCis-3a-(m-Methoxyphenyl) octahydroindole and the phenolic relative (semi-rigid phenethylamines) inhibited strongly the uptake of NE but did not cause release. Further (in vivo) studies are planned with these compounds and analogs (especially trans-isomers) to delimit structural and stereochemical requirements for the catecholamine-uptake system.

(3) cAMP Studies. Using selective H_1 and H_2 agonists and antagonists, it was shown that stimulation of the formation of cAMP in guinea-pig brain slices was greatest in the cortex and hippocampus, that receptors in the cortex were predominantly of the H_1 type and that those in the hippocampus were a fairly even mixture of H_1 and H_2. It was also observed that synergism between adenosine and histamine in the production of cAMP was mediated primarily by the H_1 histamine receptors and the affinity of these receptors for H_1 agonists is increased by adenosine (M. E. Rogers with K. Dismukes and J. W. Daly, Pharmacodynamics).

In an effort to develop selective inhibitors of cAMP phosphodiesterase (PDE), agents of potential importance as therapeutic agents and pharmacological tools, an assay for PDE activity has been set up, which measures the labelled 5'-AMP produced. If it is found that sulfhydryl (SH) groups are in or near the active site of the enzyme, compounds having affinity for the active site and that will react with SH groups to irreversibly inhibit the enzyme will be synthesized (M. E. Rogers with J. W. Daly, Pharmacodynamics).

(4) Thujone analgesia. Because Δ^9-tetrahydrocannabinol (Δ^9-THC) has analgesic activity (animals) and as it has been shown to have stereochemical and topological similarities to thujone, the latter was tested for hot-plate activity and acute toxicity (mice). It is indeed nearly as active as Δ^9-THC with a therapeutic index of 10. Several other simple terpenes, similar in structure to thujone were inactive (K. Rice and R. S. Wilson).

(5) Testing program. Analgesia, abuse liability, narcotic antagonism. As the coordinating unit for the Committee on Problems of Drug Dependence, NRC, NAS, 135 compounds from the pharmaceutical industry, universities and the synthesis program of Medicinal Chemistry, LC, have been examined in the hot-plate and Nilsen methods for analgesic activity. Interesting compounds were then tested at the Medical College of Virginia and at the University of Michigan for abuse potential (psychologic and physiologic dependence liability) and narcotic antagonist property. A few of the most promising compounds are being studied in man. Several potential codeine and morphine substitutes have emerged. Of particular interest is (-)-9β-hydroxy-9-nor-hexahydrocannabinol a (non-nitrogenous) potent analgesic without physical dependence capacity in monkeys and with pronounced hypotensive (dogs) and anticonvulsant effects (mice). This compound was synthesized by R. S. Wilson of our Section (E. L. May, L. Atwell).

(6) Role of norepinephrine (NE) in the perception of pain. Experimental in vivo work concerned with the investigation of the role of NE in the process of nociception and involving the intentionally modified levels of catecholamines and electrical stimulus as the nociceptive agent, has revealed the heretofore unknown dependence of the vocalization threshold in mice on the

endogenous concentrations of NE. The observed, directly proportional relationship indicates a fundamental role of NE in the phenomena of nociception and analgesia (L. Getsiv).

SECTION ON METABOLITES

Histrionicotoxin Analogs

The principle of 1,3-dipolar intramolecular addition has been applied successfully to the synthesis of hydroxycyclohexyl-spiro-piperidines with the essential 5-carbon side chain next to nitrogen and the less essential 4-carbon side chain missing. The isomeric hydroxymethylcyclopentyl-spiro-piperidines are also formed by this process and are being evaluated neurophysiologically. (E. Gössinger and T. Spande)

Synthesis of Dihydroadalin

Intramolecular 1,3-dipolar addition of piperidine nitrones with appropriate unsaturated side chains has made possible a new approach to the active principle present in lady bugs, adalin, and its dihydro derivative, a pseudopelletierine with a 5-carbon side chain at the bridge head. The compound resembles histrionicotoxin and has related activity on ion transport in cholinoceptors. (E. Gössinger)

Harvard Program on Histrionicotoxins

Professor Yoshito Kishi, Harvard University, has solved an important problem in logistics and supply of Perhydro-, decahydro, octohydro- and tetrahydro- histrionicotoxins, their epimers, higher and lower homologs and analogs missing one side-chain. All these compounds are now being evaluated at the Department of Pharmacology (E. X. Albuquerque) at the University of Maryland Medical School.

Radioactive Batrachotoxin and Binding Studies

A major portion of work has been devoted to the preparation of a radioactive batrachotoxin (BTX) derivative and its use in characterization of toxin binding to excitable tissues. Initially, tritiated BTX with label randomly distributed was prepared commercially by a modification of the Wilzbach procedure. The product of this procedure was purified by a series of chromatographies to a homogeneous material with a specific activity of ca. 100 mC/mM and a toxicity in mice comparable to that of unlabeled BTX. This material was used in a series of binding studies on mouse diaphragm, bovine retina and bovine serum albumin. Subsequently, a radioactive BTX derivative, the preparation of which will be described below, was used in similar studies on mouse diaphragm. The specific activity of this BTX derivative was ca. 230 mC/mM. The results of these binding studies are tentative due to the low "counts bound" to background ratio which was obtained, a consequence of the low concentration of BTX binding sites in muscle. Nevertheless, several conclusions seem warranted.

1. A high affinity, saturable, irreversible binding for BTX is present in mouse diaphragm along with a low affinity, non-specific binding, at least a portion of which is reversible.
2. This irreversible, high affinity binding is not antagonized by high concentrations (10^{-5} M) of tetrodotoxin (TTX).
3. The concentration of high affinity BTX binding sites in muscle appears to be approximately the same order of magnitude as that determined for TTX binding sites.
4. BTX did not bind to bovine serum albumin under physiological conditions (37°C, Tyrode's solution), suggesting that the mechanism of binding in muscle does not involve reaction with protein disulfide bridges.
5. Binding of BTX to retina was low and irreproducible. This is consistent with the observation that BTX is active in retina only at relatively high concentrations (E. X. Albuquerque, personal communication). Retina, although containing a high concentration of sodium channels, is therefore not suited for binding studies.

The success of these binding studies depends primarily on the availability of a radioactive BTX derivative of high specific activity, i.e. on the order of curies per millimole. Therefore, in order to investigate various derivatives, a rapid, reliable esterification procedure for BTX-A was developed. The esterification involves reaction of BTX-A with the mixed anhydride prepared from ethylchloroformate and the acid of choice and is routinely done on a 100 microgram scale. Yields are consistently 70-100%. This procedure was used to prepare the radioactive derivative mentioned above. 2,4-dimethyl-5-acetyl-pyrrole-3-carboxylic acid was reduced with tritium gas over Pd/C catalyst to 2,4-dimethyl-5-ethyl(^{3}H)-pyrrole-3-carboxylic acid, and this acid used to esterify BTX-A. The activity of this acid was increased to ca. 8C/mM by reduction with neat tritium gas (performed by New England Nuclear), but the subsequent esterification failed, presumably due to autoradiolysis effects. Therefore another, more stable derivative must be found. To this end, the p-iodobenzoate and the benzoate of BTX-A have been synthesized and are currently being tested for activity. (G. Brown)

Novel Batrachotoxins and Spin Label Studies

Other derivatives prepared by this method are of interest. 7,8-dihydro BTX was synthesized from synthetic 7,8-dihydroBTX-A (H. Wehrli) and was found to have a LD_{50} of 20 micrograms/kg (s. c. in white mice). Thus, this represents a total synthesis of a highly active BTX derivative which could be prepared on a large scale. Purely synthetic 7,8,16,17-tetrahydroBTX was also prepared, but the toxicity of this compound was only ca. 1 mg/kg LD_{50}. Also synthesized was a spin-labeled derivative starting with 2,2,5,5-tetramethyly-1-pyrrolidinyloxy-3-carboxylic acid. This compound may prove valuable in conjunction with work on the isolation of the sodium channel macromolecule. (G. Brown)

The isolation and characterization of the "sodium channel" macromolecule is being attempted in collaboration with Dr. Oliver Dolly, Dept. of Cell Biology and Pharmacology at the University of Md. Medical School. Preliminary results show that a component of rat diaphragm muscle which binds BTX is soluble in SDS and has a molecular weight of 100,000. (O. Dolly, E. X. Albuquerque and G. B. Brown)

Spin Labeling of Antigen-Binding Sites

Another collaboration was begun with Dr. C.P.J. Glaudemans to study the nature of the antigen binding site in a series of homogenous antigalactans obtained from myeloma. A spin-labeled derivative of methyl-β-D-galactoside was prepared and shown to bind to antigalactan J-539. The spin-labeled derivative was displaced by treatment with excess unlabeled methyl-β-D-galactoside, thus showing that the labeled compound binds to the same site as unlabeled. It now remains to measure the esr spectrum of bound and free spin label. The environment of the binding site, whether hydrophobic of hydrophilic, should be reflected in the shape of the spectrum of bound label. (C.P.J. Glaudemans and G. B. Brown)

Continuing Investigations on Pharmacologically Active Principles of Neotropical Frogs

Investigations of alkaloids from skin extracts of fifteen different species of dendrobatid frogs by a combination of gas chromatography-mass spectrometry (electron impact and chemical ionization techniques), coupled with isolation by thin-layer or column chromatography and high resolution mass spectrometric analysis of the isolated compound has demonstrated the occurrence of 80 different alkaloids to which many can now be assigned probable structures. Many of these compounds have interesting pharmacological activity. For example, a trace alkaloid present in at least three species has been found to elicit a Straub tail reaction in mice and to be a more potent analgesic than morphine. Another causes a sustained penile erection after subcutaneous injection in mice. Efforts to isolate sufficient quanitites of these two alkaloids for structure determination and further pharmacological investigation are in progress. Extracts from a variety of other frogs under investigation. A water-soluble toxic substance has been detected in extracts from *Colostethus inguinalis* and a chloroform-soluble neutral toxin in extracts from *Atelopus ignescens*. Alkaloids have been detected only in extracts from dendrobatid frogs. A new dihydrohistrionicotoxin containing an acetylenic moiety in the five carbon side chain has been isolated and is under pharmacological investigation. (J. W. Daly, C. W. Myers, T. Tokuyama)

Crystallization of Antamanide and its Metal Complexes for X-ray Conformational Analysis

Antamanide is a cyclic peptide which counteracts the liver toxin, phalloidin, presumably by tightening the liver cell membrane agains penetration of the toxin. Physico-chemical investigations have revealed that antamamide selectively forms complexes with sodium and calcium ions (less strongly also with lithium ions), and that several conformations of the peptide occur in solvent and cation dependent equilibria. X-ray analyses of the lithium- and sodium-antamamide complexes have been completed. Further x-ray analyses of active and inactive antamanide derivatives and their complexes especially with calcium ions are expected to contribute to an understanding of the biological action of antamanide. Biologically inactive perhydro-phe^4-val^6-antamanide was crystallized from metal ion free solutions as well as with lithium, sodium, and calcium ions being added. Active phe^4-val^6 antamanide was crystallized alone from calcium ions containing solutions. X-ray analysis of the crystal samples is under investigation in the laboratory of Dr. J. Karle, Naval Research Laboratory, Washington, D. C. (W. Burgermeister)

Radioactive Labeling of Inhibitors of the Choline Receptor

Tropane alkaloids, such as scopolamine and atropine, bind competitively to the muscarinic acetylcholine receptor. Local anaesthetic tropeines, such as cocaine, inhibit excitatory ion conductance changes of the nerve membrane. The frog toxin, histrionicotoxin, interacts with ion conductance modulator sites of the motor endplates. Radioactive tropeines with high specific activity are being prepared which could be used for the isolation of the muscarinic acetylcholine receptor from tissues (in collaboration with Dr. Marshall Nirenberg, NHLI), for the characterization and planned isolation of ion conductance modulator structures, and for the differentiation of the biological effects of local anaesthetic tropeines and histrionicotoxin. In the course of these ongoing preparations scopolamine was demethylated to norscopolamine. The latter was remethylated with tritium-methyliodide. Tritiated scopolamine with an activity of 11 curies per millimole was obtained and purified by thin layer chromatography. Similar radioactive labeling is in progress with other tropeines and with such derivatives that have a structural relation to histrionicotoxin. (W. Burgermeister)

Failure of Duplexes Based on Polylaurusin [Poly(L), "Polyformycin B"] To Induce Interferon

Polylaurusin[poly(L) or "polyforymicin B"] forms double-stranded complexes with polycytidylic acid (poly(C)) and with poly(5-bormocytidylic acid) [poly(br^{5}C)] with T$_m$'s of 46.5° (0.2 *M* NaCl, pH 7) and 72.5° (0.15 M NaCl, pH 7), respectively. Both complexes fail to provide antiviral resistance (against vesicular stomatitis virus in primary rabbit kidney cells) or to induce interferon in "superinduced" primary rabbit kidney cells, even though they fulfill all previously recognized requirements for effective interferon inducers. The studies again suggest that the first step in interferon induction may be the recognition of a particular RNA conformation by a cellular receptor site. (P. F. Torrence, E. De Clercq, J. A. Waters, B. Witkop)

Inhibition of Oncornavirus Functions by 2'-Azido Polynucleotides

The 2'-azido analogs of poly(U) and poly(C), poly(dUz) [poly(2'-azido-2'-deoxyuridylic acid)], and poly(dCz) [poly(2'-azido-2'-deoxycytidylic acid)], were found to inhibit the RNA-directed DNA polymerase (reverse transcriptase) activity of murine leukemia (Moloney, Rauscher) and sarcoma (Moloney) virus, and feline leukemia (Theilen) and sarcoma (Gardner) virus, while under the same conditions the unsubstituted parent compounds failed to do so. In addition, poly(dUz) and poly(dCz) inhibited the replication of *exogenous* murine sarcoma virus (Moloney) in nontransformed cells (as assessed by an infectious center assay), but poly(dUz) failed to suppress the formation of *endogenous* sarcoma and leukemia viruses in transformed cell lines (MO-P, JLSV5). In these same cells, poly(dUz) failed to inhibit the multiplication of vesicular stomatitis virus. These data add further strength to the contention that reverse transcriptase is necessary for the productive infection and transformation of normal cells by oncornaviruses but is not essential for the maintenance of this transformed state and the continuous production of new viruses particles by these transformed cells. (E. De Clercq, A. Billian, J. Hobbs, P. F. Torrence, B. Witkop)

Biological, Biochemical, and Physicochemical Evidence for the Existence of the Polyadenylic·Polyuridylic·Polyinosinic Acid Triplex

When primary rabbit kidney cell cultures are treated with either polyadenylic acid·polyuridylic acid or polyadenylic acid·polyribothymidylic acid (poly(rT)) and then judiciously exposed to actinomycin D and cycloheximide, high titers of interferon are found in the extracellular medium ("super-induction") (Vilcek, J. (1970) *Ann. N. Y. Acad. Sci.* 173, 390-403; Tan, Y. H., Armstrong, J. A., Ke, Y. H., and Ho, M. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 67, 464-471). If polyinosinic acid is added 1 hour prior to, simultaneously with, or 1 hour after the active interferon inducers, dramatic reductions in interferon production from the "superinduced" cells result. Based on experiments involving sucrose gradient ultracentrifugation, pancreatic ribonuclease A resistance, ultraviolet mixing curves, and ultraviolet absorbance-temperature profiles, the explanation for this phenomenon was determined to be the formation of polynucleotide triplexes in the following way:

poly(A)·poly(U) + poly(I)   poly(A)·poly(U)·poly(I)

poly(A)·poly(rT) + poly(I)   poly(A)·poly(rT)·poly(I)

In addition, based on similar methodology, the following reactions involving these triplexes were demonstrated:

poly(A)·2 poly(I) + poly(U)   poly(A)·poly(U)·poly(I) + poly(I)

poly(A)·2 poly(I) + poly(rT)   poly(A)·poly(rT)·poly(I) + poly(I)

poly(A)·2 poly(I) + 2 poly(U)   poly(A)·2 poly(U) + 2 poly(I)

and

poly(A)·poly(U)·poly(I) + poly(U)   poly(A)·2 poly(U) + poly(I)
(E. De Clercq, P. F. Torrence, P. De Somer and B. Witkop)

Antiviral Activity of 5-Thiocyanatopyrimidine Nucleosides

The antiviral activity of 5-thiocyanatouridine (5-SCNrU), 5-thiocyanato-2'-deoxyuridine (5-SCNdU), 5-thiocyanatouracil arabinoside and 2',3',5'-tri-O-acetyl-5-thiocyanatouridine has been evaluated in primary rabbit kidney cell cultures challenged with either DNA (vaccinia, herpes simplex) or RNA (vesicular stomatitis) viruses. 5-SCNdU was particularly effective against DNA viruses, reducing vaccinia virus replication and cytopathogenicity at concentrations as low as 4-10 g/ml. The tri-O'-acetylated derivative of 5-SCNrU inhibited the infectivity of vesicular stomatitis virus but only at relatively high concentrations (100 μg/ml). The inhibitory effect of 5-SCNdU on DNA virus multiplication was not paralleled by an inhibition of host cell DNA synthesis, as monitored by incorporation of ^{3}H-thymidine. In fact, 5-SCNdU caused a significant (up to 30-fold) increase of ^{3}H-thymidine incorporation at concentrations up to 200 μg/ml. RNA synthesis (as measured by ^{3}H-uridine incorporation) was not markedly affected by either of the compounds tested. These results justify further trials with 5-SCNdU in experimental DNA virus infections in animals. (E. De Clercq, P. F. Torrence, J. A. Waters, B. Witkop)

Antiviral and Antimetabolic Activities of Poly(7-deazaadenylic acid) and Poly(7-deazainosinic acid)

The antiviral and antimetabolic activities of poly(7-deazaadenylic acid) [poly(c^{7}A)], poly(7-deazainosinic acid) [poly(c^{7}I)] and their nucleosides 7-deazaadenosine (c^{7}A) and 7-deazainosine (c^{7}I) have been assessed in primary rabbit kidney (PRK) and mouse JLSV5 cells, the latter being a transformed, (Rauscher) murine leukemia virus-shedding, cell line. The antiviral activity was monitored by investigating the effects of the compounds on the replication of vaccinia, herpes simplex or vesicular stomatitis virus in PRK or JLSV5 cells and by measuring endogenous oncornavirus production in JLSV5 cells. The inhibition of cellular RNA and DNA synthesis served as parameters of antimetabolic activity. With all compounds tested the minimum effective concentrations inhibiting virus replication coincided remarkably well with the minimum toxic concentrations impairing cellular RNA and DNA synthesis or normal cell morphology. These concentrations amounted to 0.3-0.6 μg/ml for c^{7}A and poly(c^{7}A), 3 μg/ml for c^{7}I and 10 μg/ml for poly(c^{7}I). At higher concentrations (∿ 70 μg/ml) poly(c^{7}A) and poly(c^{7}I) were also found to inhibit in vitro DNA polymerase activity of (Moloney) murine leukemia virus. According to the data presented herein, poly(c^{7}A), poly(c^{7}I) and the corresponding monomers c^{7}A and c^{7}I cannot be considered as specific antiviral or antitumor agents. (E. De Clercq, A. Billiau, P. F. Torrence, J. A. Waters, B. Witkop)

Sustained Action Forms of Biologically Active Nucleosides

Synthetic modifications of antitumor, antiviral and immunosuppressant nucleosides are being studied for (1) the promotion and (2) the maintenance of effective therapeutic levels of the nucleoside within the cell. In an attempt to achieve these objectives, selective esterifications of the 5'-hydroxyl group of several candidates, i.e., 5-FdU, 5-IdU and AraC, with 2,2,6-trimethylcyclohexane-1-carboxylic acid are being carried out. Cellular enzymatic cleavage, most likely by esterases, should slowly release the compound in its active form. (J. A. Waters & G. Polansky).

Analgesics Based on the Piperdine Ring System

Analgesic compounds have been synthesized from 4-piperidinol. These compounds do not possess a quaternary carbon atom. Of the compounds evaluated thus far, 4-hydroxy-N-methylpiperidine pyrrole carboxylate exhibits a potency intermediate between that of codeine and morphine in the hot plate assay, shows no morphine-like physical dependence capacity in monkeys and is long acting. Structural features at various positions of the ring system and analgesic receptor affinities will be examined. (J.A. Waters & G. Polansky).

Stereospecific Acid-Catalyzed Rearrangement of 1,6-Dimethylpentacyclo[6,4,0,-$0^{2,7}$,$0^{3.10}$,$0^{6,9}$]dodeca-5,12-dione to a Bisnordiadamantane

In continuation of photochemical studies in collaboration with the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan and the Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C., 1,6-dimethylpentacyclo[6,4,0,$0^{2,7}$,$0^{3,10}$,$0^{6,9}$]dodeca-5,12-dione has been treated with trifluoracetic acid or para-toluenesulfonic acid to give the bisnordiadamantane 1,5,6,6,12,12-hexamethylpentacyclo[6,3,1,$0^{2,7}$,$0^{3,10}$,-$0^{5,9}$]dodecane-4,11-dione, through two sets of stereospecific two-fold Wagner-Meerwein rearrangements in almost quantitative yield. (K. Hirao, M. Taniguchi, O. Yonemitsu, J. L. Flippen, I. L. Karle & B. Witkop).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Function and Instrumentation

Approximately 30 elements and five functional groups are determined as a service function to investigators of the laboratory by the microanalytical service. The total analyses performed for the year were 13,427, a 20% increase over the previous year. Routine instrumental service continues in the areas of IR, NMR, GLC, and GC/MS. The gas chromatography service has been upgraded by the addition of a radioactive monitoring system (RAM-G. D. Searle) (H. Yeh, N. Whittaker, W. Landis, B. Miller, P. Parisius, A. Wong, B. Baer, and D. F. Johnson).

The Study of Steroid Transformations by Tetrahymena Pyriformis

Previous studies have demonstrated that a variety of steroids of mammalian origin as well as steroid derivatives such as dialkylaminoethoxy steroids inhibit the growth of the protozoa, T. pyriformis, and that this growth inhibition is reversed by the addition of cholesterol. The mode of action of these growth-inhibitory steroids is not definitely known, but inhibition of NADH oxidation and RNA synthesis, and interference with respiration and formation of lipids have been demonstrated. T. pyriformis grown without added steroids produces a pentacyclic triterpenoid alcohol, tetrahymenol as its major unsaponifiable product by a direct, non-oxidative, proton initiated cyclization of squalene. When steroids such as dehydroepiandrosterone are added to cultures, an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. Since the focus of attention has been on the action of steroids on Tetrahymena, the present studies are designed to study the effect of the protozoa on the steroid molecule. All of the substrates incubated (androstenedione, progesterone, 17α-OH-progesterone, testosterone, corticosterone, and pregnenolone) have yielded metabolites, as indicated by column and thin-layer chromatography. The most frequently observed transformation indicated by thin-layer chromatographic analysis has been the reduction of a 3-ketone to a 3β-hydroxyl. This transformation by T. pyriformis has been confirmed by the identification of 4-androstene-3β,17β-diol as a product from testosterone incubations. The identification of several other radioactive products is being pursued in the hope of establishing that T. pyriformis can isomerize a Δ^4-steroid to a Δ^5-steroid, as well as reducing a carbonyl.

Isolation of 4-androstene-3β,17β-diol as a transformation product from testosterone incubation with T. pyriformis indicates that this protozoan has a β-hydroxysteroid dehydrogenase system. An attempt is being made to isolate and characterize this enzyme system (N. Lamontagne and D. F. Johnson).

Gonadal Steroid-Stimulated Formation of Cyclic AMP in Incubated Rat Hypothalmus

In the immature female rat, diethylstilbesterol (DES) and estradiol (E_2) elicit significant increases in levels of endogenous and [^{14}C]cyclic AMP in [^{14}C]adenine-label hypothalami. The possible link between the estrogens, catecholamines and cyclic AMP accumulation was studied in vitro. Similar considerations apply to the immature male rat.

As mentioned above, DES and E_2 but not estriol or 17α-estradiol elicited increased cyclic AMP formation. This stimulation could be blocked by α and β adrenergic blockers and the anti-estrogen clomiphene in the female rat. It was also found that higher doses of DES has a dual effect: a) estrogenic and b) possibly a release of adenosine. The accumulation of cyclic AMP could also be blocked in the female rat by slicing the hypothalamus and adding theophylline. In the case of male rats, testosterone and DHT, the major androgens, have no significant effect on the adanylate-cyclase system while E_2 has a small effect. On the other hand, DES is very potent in this respect and cannot be blocked by α or β adrenergic blockers as well as dopamine antagonists. Clomiphene too has no effect on the stimulated levels of cyclic AMP elicited by DES (B. A. Weissman, P. Skolnic, D. F. Johnson, and J. Daly).

Nuclear Magnetic Resonance Studies

In addition to some 500 specialized NMR spectra on the Varian HA-100 and 50 on a Varian HR-200 as a service function, a number of collaborating studies have been carried out (H. Yeh) in this laboratory. The following list summarizes some of the studies completed or in progress:

1. Applications of NMR to the Structural Elucidation of Synthetic Drugs, Metabolites and Natural Products (A. E. Jacobson, R. S. Wilson, and E. L. May).

2. NMR Studies of Reaction Rate, Molecular Motion and Molecular Interaction in Biochemical and Biological Systems (A. M. Jeffery, D. M. Jerina, H. Ziffer, and T. F. Spande).

3. A Study of the Interaction between Organic Phosphates and Biogenic Amines by ^{31}P NMR (R. Katz and D. F. Johnson).

4. ^{19}F and ^{1}H NMR Studies of Ring-Fluorinated Imidazoles and Histidines (K. Kirk, L. Cohen, and J. S. Cohen).

5. NMR Study of Benzene Oxide-Oxepin Tautomerism (H. Yagi and D. M. Jerina).

6. An NMR Study of Equilibria and Paramagnetism in a Ferriprotoporphyrin-Cyanide System (J. T. Wang and D. F. Johnson).

The Development of Chemical Methods and the Synthesis and Study of Chemical Compounds of Use in the Inestigation of Biological and Medical Problems.

The primary goal of this project is to make contributions to the investigation and solution of basic biological and medical problems. A substantial expenditure of effort has been and is being made in the area of establishing and maintaining an adequate file of notes, references, reprints, etc. to make possible and facilitate future and current research.

Another goal of this project is to extend the earlier work on the photocyclization reaction observed with N-chloroacetyl-L-tryplophan to other systems which may yield compounds of chemical and biological interest (C. Foltz).

SECTION ON OXIDATION MECHANISMS

Major Findings: Efforts have continued toward establishing mechanisms and pathways of metabolism of aromatic compounds by enzymatic systems. Pseudomonas putida was demonstrated to stereospecifically form a cis 1,2-dihydrodiol from naphthalene through the action of a dioxygenase based on incorporation of both oxygen atoms from $^{18}O_2$. Both mammalian systems and bacteria convert dihydrodiols to catechols via dehydrogenases. Deuterium labeling studies established that the bacteria dehydrogenase functions by simultaneous removal of both carbinol hydrogens while the mammalian enzyme behaves more like a typical alcohol dehydrogenase. A mutant species of Beijerinckia converts both benzo[a]pyrene (BP) and benzo[a]anthracene to mixtures of cis dihydrodiols. The diols from BP have been identified as cis-7,8-dihydroxy-7,8-dihydro BP by comparison with authentic standards for which chemical routes of synthesis were devised. Two lines of evidence have been pursued which indicate that arene oxides may not be the sole mechanism by which phenols are formed from xenobiotic substances in mammals: i) the first instance primary kinetic isotope effects have been detected, and ii) ratios of phenolic metabolites have been found which are inconsistent with arene oxide intermediates. Metabolism by solubilized and purified hemeproteins (P-450 and P-448) have indicated that cytochrome b_5 and cytochrome b_5 reductase may well play a role in the non-arene oxide pathway proposed. If such a non-arene oxide pathway could be made prominent by induction, the toxic arene oxide pathway could be avoided in mammals. (Jeffrey, Yeh, Yagi, Selander, Tomaszewski, Daly, and Jerina).

Explorations into the fundamental chemistry of arene oxides and polycyclic hydrocarbons have advanced. A multi-step synthesis of 1-hydroxybenzo[a]pyrene has been completed in order that this compound can be tested as a potential carcinogen and mutagen. Reaction of a variety of nucleophiles with benzene oxide has shown that attack occurs not only by simple 1,2-opening, but also by 1,4- and 1,6-attack. The stereochemical course of these reactions is dependent on the nature of the nucleophile. Comparison of the solvolytic chemistry of K-region and non-K-region arene oxides has shown that trans dihydrodiols rather than phenols are formed from K-region arene oxides at physiological pH. This accounts for the failure of previous workers to detect K-region phenols as metabolites from polycyclic hydrocarbons. Careful examination of the structure of mercapturic acid adducts from arene oxides has shown much of the previous literature on this subject to be in error due to unprecedented migrations of the thiol residues on dehydration and aromatization. Photolysis of arene oxides at low temperature ($-196^{\circ}C$) has established that keto tautomers of phenols are initial photoproducts. The keto tautomers are of interest since they are precursors to the phenols formed from arene oxides in biological systems. Several years of effort on the synthesis of K-region and non-K-region arene oxides have culminated in the development of highly efficient and convenient methods for the preparation of these compounds. Both types of arene oxides are prepared from halohydrin ester precursors which serve as masked functionality for the oxirane ring (Lindsay-Smith, Jeffrey, Yeh, Yagi, Selander, and Jerina).

The mobilities of 24 potential metabolites of benzo[a]pyrene were examined with high pressure liquid chromatography. Twelve phenols, five quinones, four dihydrodiols, and three oxides were studied. The chromatographic procedure employed allowed the separation and quantitation of benzopyrene metabolites into three major groups consisting of phenols, quinones, and dihydrodiols. Two of the benzopyrene oxides were unstable during chromatography whereas the third oxide was more stable and chromatographed in the quinone fraction. Treatment of rats with phenobarbital or 3-methylcholanthrene enhanced the metabolism of benzopyrene by liver microsomes and altered the relative amounts of the various metabolites formed. In the absence of epoxide hydrase (EC 4.2.1.63), benzopyrene was metabolized primarily to phenols and quinones but was not appreciably metabolized to dihydrodiols by a solubilized reconstituted cytochrome P-448 monooxygenase system. Addition of partially purified epoxide hydrase resulted in the formation of benzopyrene dihydrodiols with a concomitant decrease in the formation of phenolic metabolites, indicating that benzopyrene undergoes metabolism via arene oxides that are precursors for dihydrodiols and phenols. The above study was made possible because of a prior investigation in which epoxide hydrase and cytochrome P-450 were separated by chromatographic procedures (Holder, Dansette, Yagi, and Jerina).

SECTION ON PHARMACODYNAMICS

Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

More efficient methods for the isolation of catechol-O-methyl transferase (COMT) utilizing affinity and immunoabsorption chromatography have been developed. The amino acid composition of one form of COMT, mol. wt. 23000, has been determined. The interaction of 6-hydroxydopamine with one of the two sulfhydryl groups present in the active site of COMT has been demonstrated. A radioimmunoassay for COMT has been perfected and used to measure enzyme levels in discrete areas of rat brain. The localization appears to be similar in certain respects to the localization obtained with antibody-peroxidase studies. The peroxidase localization appears to be restricted to membrane-bound forms of COMT and in preparations of rat cortex the loci appear as widely distributed arc-like structures which may be similar to the apparent dendritic terminals outlined by immunofluorescent techniques. Electron microscopic studies with ferritin-labeled anti-COMT and highly purified synaptosomes from rat brain tentitatively confirm the localization of membrane-bound COMT at the post-synaptic membrane. Several new approaches to improving the specificity of the ferritin-binding using isolated liver microsomes as a tissue substrate have been developed. Radioactive ferritin was prepared and used to quantitate the binding and negative staining techniques were used to observe the specificity of the binding. Washing the tissue preparation with a lysine buffer markedly reduces the nonspecific binding.

Studies on the structure/activity correlations on the inhibition of uptake, release and long term effects on the uptake mechanisms by phenethylamines, phenethanolamines and indoleamines in heart have been continued. A technique for measuring the uptake of amines by diced suspensions of mouse atria has been developed which includes several technical improvements such as the simultaneous determination of the uptake of tritiated amines and the atrial sample size with the diffusion of carbon-labeled inulin and the essentially instantaneous termination of uptake by the addition of cold fixative to the reaction tube. Control of the amine concentration in this *in vitro* assay is much more precise than in previous studies which were performed *in vivo* and permits more accurate estimations of the affinity of adrenergic neurons for amines. Studies of the uptake of tritiated 6-hydroxydopamine and the irreversible binding of the oxidation products of this amine have clearly shown that there is a rapid onset of irreversible binding and a concommitant loss of the uptake of 6-hydroxydopamine and norepinephrine. Similar studies have been carried out with tritiated 5,7-dihydroxytryptamine in an attempt to gain some insight into the observation that prior inhibition of MAO blocks the loss of the uptake mechanism and the ensuing neurodegeneration usually seen with 5,7-dihydroxytyrptamine.

Routine studies on the inhibition of norepinephrine uptake and the release of norepinephrine are being continued in an attempt to gain further knowledge of the specificity of the uptake mechanism and to find additional compounds which may be useful as investigational tools. Currently we are examining the fluoro derivatives of tyramine and dopamine in this regard. 3-Fluoro tyramine and 6-fluorodopamine are substrates for uptake and displace endogenous norepinephrine.

To examine the effects of 6-hydroxydopamine8with regard to mechanism of neurocytotoxicity and to search for model systems of the active uptake mechanism studies were performed in neuroblastoma and other cell cultures. Facilitated uptake of dopamine and 6-hydroxydopamine were obtained in an adrenergic clonal line of neuroblastoma, N1E-115, but no clear evidence was obtained for active uptake. Determination of the extent of total protein binding of 6-hydroxydopamine and the nature of the soluble proteins extracted from these cells revealed that one or more of the oxidation products of 6-hydroxydopamine were covalently bound to protein. There was a marked increase in the proportion of high-molecular weight proteins in these cells and these proteins contained nearly all of the bound 6-hydroxydopamine. This result supports our previous hypothesis derived from model studies with purified proteins in vitro that the mechanism for neurocytotoxicity results from the intraneuronal cross-linking of proteins by one or more of the oxidation products of 6-hydroxydopamine.

A study of the incorporation in protein, distribution and metabolism of ^{3}H-2-fluoro-histidine in mouse is being continued. Incorporation of 2-fluorohistidine into globin in vitro utilizing the rabbit reticulocytic system has been demonstrated. Studies are in progress which indicate that 2-fluorohistidine can substitute for histidine giving rise to globin containing 2-fluorohistine.

Evidence for direct sympathetic innervation of thyroid follicular cells has been obtained by morphological studies of mouse thyroids in conjunction with studies on the uptake of 6-hydroxydopamine, 5,7-dihydroxytryptamine and 5-hydroxydopamine in vivo.

The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

Methoxamine has been found to be a specific stimulant of α-adrenergic receptors controlling cyclic AMP formation in rat cortical slices. The magnitude of responses of cortical cyclic AMP-generating systems to methoxamine correlated negatively with spontaneous behavioral activity in four strains of rats. Certain of the α-receptors appear activated by norepinephrine only in the presence of adenosine. In rat cortical slices both norepinephrine and methoxamine will elicit repetitive accumulations of cyclic AMP. Thus, the refractoriness to amines after one stimulation which pertains in guinea pig does not pertain in rat. (Skolnick)

Clonidine, normally considered a potent α-agonist, has been found to be perhaps the most active known α-antagonist towards norepinephrine-stimulated formation of cyclic AMP in rat cortical slices. In addition, clonidine, oxymetazoline and phenylephrine greatly potentiate the accumulation of cyclic AMP elicited by very low concentrations of the β-agonist, isoproterenol (Skolnick).

In rat cerebellar slices, β-antagonists completely block the accumulation of cyclic AMP elicited by norepinephrine or isoproterenol. Neuroleptics also antagonize the effect of the catecholamines but only by 30-40%. It would appear based on these results and parallel studies on the prevention by β-antagonists and neuroleptics of the inhibitory effects of catecholamines on spontaneous discharge of cerebellar Purkinje cells that norepinephrine-elicited formation of cyclic AMP occurs in at least two cell populations and that only the β-receptors of Purkinje cells are blocked by neuroleptics such as flupenazine and α-flupenthixol (Skolnick).

Histamine-stimulated formation of cyclic AMP in guinea pig cortical slices has been shown to occur primarily through activation of an H_1-receptor, while in hippocampus both H_1- and H_2-receptors are important.

Synergism between histamine and adenosine in both brain regions occurs primarily through H_1-receptors. The affinity of histamine analogs for the H_1-receptors is greatly increased by adenosine. Metiamide was used in these studies as a H_2-antagonist, d-brompheniramine and other classical antihistamines as H_1-antagonists, 4-methylhistamine as an H_2-agonist and 2-aminoethylthiazole as an H_1-agonist (Rogers, Dismukes).

Prostaglandins of the E series were shown to stimulate accumulation of cyclic AMP in rat brain slices (neocortex, striatum, midbrain-thalamus-hypothalamus), while prostaglandin $F_{1\alpha}$, A_1, and B_1 were ineffective. Relatively high concentrations of the prostaglandin (EC_{50} ~ 20μM) were required and no antagonists were found. Indeed, morphine potentiated the effect of the prostaglandin. Prostaglandin E_1 appeared to antagonize the accumulation of cyclic AMP elicited by β-adrenergic agonists (Dismukes).

Radioactive 2-chloroadenine was not effectively incorporated into intracellular nucleotide pools of guinea pig cortical slices and no formation of radioactive 2-chlorocyclic AMP could be detected during subsequent stimulations. Thus, of the various adenine and adenosine analogs studied as potential precursors of abnormal cyclic nucleotides in brain slices only 2'-deoxyadenosine led to formation of such a nucleotide, 2-deoxycyclic AMP (Mah).

Glutamate stimulates cyclic AMP formation in guinea pig cortical slices, but relatively high concentrations are required and the response is blocked by the adenosine antagonist, theophylline. Results obtained with other adenosine antagonists, (isobutylmethylxanthine, adenosine deaminase) support the conclusion that glutamate stimulates cyclases in brain slices through adenosine-dependent mechanisms. However, the weak adenosine antagonist, 2-deoxyadenosine, does not block the glutamate-response. Adenine xylofuranoside appears to block the stimulatory effects of all agents tested including biogenic amines. The potentiation of responses to amines by phosphodiesterase inhibitors appears with diazepam, chlordiazepoxide, papaverine and RO 20-1724 due in part to inhibition of adenosine reuptake into slices, thus, leading to synergistic adenosine-amine interactions. (Mah)

Basal levels of cyclic AMP and cyclic GMP in guinea pig cortical and cerebellar slices appear dependent on the presence or absence of calcium ions (Ca^{++}) in the incubation medium. Levels of cyclic GMP decrease 2-3 fold in the absence of Ca^{++} in both cortical and cerebellar slices. Readdition of Ca^{++} leads to a rapid increase in cyclic GMP. Further increases can be elicited by a depolarizing agent, veratidine. The increases in cyclic GMP levels in cerebellar slices are not antagonized by theophylline, atropine, diethylglutamate, or strychnine, but are partially blocked by the GABA antagonist, picrotoxin, and to a lesser extent by bicuculline. In cortical slices, absence of Ca^{++} appears to increase adenosine efflux leading to enhanced basal levels of cyclic AMP and synergistically enhanced responses to norepinephrine. Both basal and norepinephrine-elicited levels of cyclic AMP in Ca^{++}-free medium are reduced by the adenosine-antagonist, theophylline. (Ohga)

After neonatal treatment of rats with 6-hydroxydopamine, noradrenergic innervation of cortex does not develop, while abnormal noradrenergic hyperinnervation of midbrain occurs. The accumulation of cyclic AMP elicited by norepinephrine is enhanced in both brain regions. In cortex this apparently represents a phenomenon similar to that of denervation hypersensitivity, while in midbrain the enhanced response to norepinephrine indicates that the abnormal noradrenergic terminals are indeed functional; i.e., they are associated with norepinephrine-sensitive postsynaptic adenylate cyclases. In midbrain of adult rats in contrast to cortex, destruction of noradrenergic terminals by intraventricular 6-hydroxydopamine or reduction of norepinephrine by reserpine does not lead to "denervation-like" hypersensitivity to norepinephrine. (Dismukes)

In incubated intact rat hypothalami, diethylstilbestrol elicits after a latent period of 40 min a 3-fold accumulation of cyclic AMP. This accumulation is blocked by the estrogen antagonist, clomiphene, and by the catecholamine antagonists, phenoxybenzamine, propranolol and haloperidol. An additional nonspecific stimulatory effect seen at high concentrations of diethylstilbestrol is apparently mediated by adenosine since it is blocked by theophylline. Low concentrations of estrogenic agents do not elicit accumulations of cyclic AMP in slabs of cerebral cortical tissue or in sliced hypothalami. The results suggest that estrogens evoke after a latent period a release of catecholamines which stimulate postsynaptic cyclases and that this sequence involves intact intrahypothalamic neurones. (Skolnick, Weissman)

Preliminary studies demonstrate that lesions of the medium forebrain bundle in rats results in enhanced responses of both norepinephrine and histamine-sensitive cyclic AMP-generating systems in cerebral cortical slices in the lesioned hemisphere. (Dismukes, Ghosh, Creveling)

Project No. Z01 AM 19000-14 LC
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

Previous Serial Number: NIAMDD-LC-6

Principal Investigator: R. K. Ness

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To devise methods of synthesizing the *cis* form (C-2 - C-3) of D-fructofuranosides-initially preparing the nucleoside 9-β-D-fructofuranosyladenine.

Present Status: Several approaches to securing a fructofuranose derivative with a non-participating group on the C-3 position are being attempted. One of these approaches involves the preparation of 3-O-benzyl-D-fructose, which has been converted to the methyl 3-O-benzyl-D-fructofuranoside; thence to the benzoylated product followed by hydrolysis to 1,4,6-tri-O-benzoyl-3-O-benzyl-D-fructofuranose. This will be p-nitrobenzoylated in the aromeric position for ready conversion to the halide, which will then be condensed wtih N-benzoyladenine. A second approach involves the hydrolysis of acetylated inulin to hexaacetyl difructofuranose dianhydride, which has now been benzylated. The hydrolysis of this product to 3,4,6-tri-O-benzyl-D-fructofuranose will be attempted, followed by p-nitrobenzoylation to 1,2-bis-O-(p-nitrobenzoyl)-3,4,6-tri-O-benzyl-D-fructofuranose. This would then be corverted to the halide (at C-2) and condensed with N-benzoyladenine.

Significance to Biomedical Research and to the Program of the Institute.

Psicofuranine is a ketohexosyladenine with the β-D-*ribo* configuration in the sugar ring. It is an antibiotic with marked antibacterial and antitumor activity *in vivo*. It is however also very toxic to man. The initial target compound is an epimer of psicofuranine at C-3 of the sugar, possessing a β-D-*arabino* configuration. It is anticipated that the antibiotic properties will be retained with hopefully a decrease in toxicity to humans. It is interesting to note that although fructose occurs naturally in the widely occurring β-D-fructofuranosides sucrose and inulin, β-D-fructofuranosides have been difficult to prepare chemically-sucrose itself has been chemically synthesized in very low yields on only two occasions. A good method of synthesizing β-D-fructofuranosides is needed to make such derivatives of fructose available for biomedical investigations.

Keyword Descriptors: β-D-Fructofuranosides, 9-β-D-fructofuranosyladenine, 3-O-benzyl-D-fructose, 1,4,6-tri-*O*-benzoyl-3-*O*-benzyl-D-fructofuranose, Difructofuranose dianhydride hexaacetate, Hexabenzyl-difructofuranose dianhydride.

Project No. Z01 AM 19001-03 LC
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunochemistry and Reaction of Carbohydrates

Previous Serial Number: NIAMDD-LC-7

Principal Investigator: C. P. J. Glaudemans

Other Investigators: M. Pokorny, B. Manjula, J. Tomasic, E. Zissis

Cooperating Units: M. Potter (NCI)
M. Vrana (NCI)

Man Years:

Total: 3
Professional: 3
Other: 0

Project Description:

Objectives: Homogeneous immunoglobulins can be obtained from plasma tumors. The study of their interaction with polysaccharide antigens will reveal much about the nature of action of antibodies and about their structure. We are involved in such studies.

Methods Employed: The usual techniques of chemistry and biochemistry are employed, such as affinity chromatography employing hapten-coupled sephacose columns, fluorescence titration, organic synthesis, gas-liquid chromatography, mass spectroscopy, etc.

Major Findings: 1. New Antigalactans. The group of homogeneous immunoglobulins with antigalactan specificity is now composed of 8 members. T601 has been found to be very much like X24 when comparing its association constants (K_a) with various ligands. Also there is cross reactivity between T601 and anti-X24 mouse serum. The idiotypic behaviour of T601 is however unique.

2. Specificity. The homogeneous antigalactan-immunoglobulins have shown specific interactions towards β(1→6) linked D-galactopyranosyl units. However, a galactan from the bark of white willow, having β1,4 linked galactosyl residues can be accommodated in the combining site. Therefore D-galactopyranosyl β1,4-D-galactose was synthesized and this disaccharide did cause the tryptophanyl fluorescence of J539 immunoglobulin to be enhanced. Binding studies are in progress.

3. Recombinant studies. Immunogolbulins can be reduced and alkylated (to break disulfide bonds and prevent recombination) and then separated into L and H chains (in 6M urea) by chromatography. When T601 (homogeneous anti-galactan) was so treated H and L chains could be obtained which recombined in high yield to form an immunoglobulin molecule which after chromatographic purification was indistinguishable from the native T601 (by ligand probing). With this technique in hand a number of recombinants have been prepared of the following immunoglobulins: J539 with X24 and T601 with X24. The hybrid immunoglobulins $H^{539}L^{24}$, $H^{24}L^{539}$, $H^{601}L^{24}$ and $H^{24}L^{601}$ obtained have been studied with ligands as well as for their idiotypic behavior .

It was shown that all the hybrid immunoglobulins bound Gal β1,6 Gal with a binding constant very similar to that of native anti-galactans. Other ligand probes (Diisopropylidene galactosyl β1,6 galactose, methyl 6-O-acetyl-β-D-galactopyranoside, etc.) also bound to these hybrid recombinants in a way which suggested a complete viable combining site.

Sheep red blood cells coated with arabino-galactan polysaccharides were agglutinated by the $H^{24}L^{601}$ recombinant nearly as well as did the native X24. The recombinant $H^{601}L^{24}$ was not as effective and also not as effective as native T601.

The idiotypic specificity of recombinants was tested in a number of systems, for instance X24-coated SRBC with anti-X24 antiserums was used to test the inhibitory power of recombinants as well as J539-coated SRBC with anti J539. From the results (see below) it is clear that these recombinant antibodies have an idiotypic specificity which is very close to that of the parent of the heavy chain.

Protein	X24-SRBC/anti-X24	J539-SRBC/anti-J539
	No. of wells protein dilution still showing inhibition	No. of wells protein dilution still showing inhibition
X24	8+	0
$H^{24}L^{539}$	5+	0
J539	0	8+
$H^{539}L^{24}$	0	6+

In the case of the X24/T601 hybrid antibodies the results were not as clear cut.

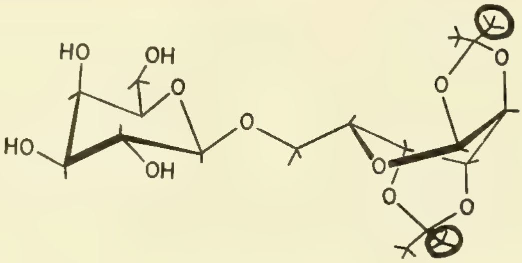
Protein	X24-SRBC/anti-24 No. of wells protein dilution still showing inhibition.	T601-SRBC/anti-601 No. of wells protein dilution still showing inhibition.
$H^{601}L^{24}$	4+	12+
$H^{24}L^{601}$	14+	9+
X24	14+	9+
T601	0	12+

4. Binding studies. Previously, we have shown that the 5-(hydroxymethyl) group of methyl β-D-galactopyranoside does not make a large contribution to the binding of that ligand with IgA J539 and X24 (Fab'). We have now shown that a relatively bulky group, such as the acetyl group, in no way hinders the binding of methyl 6-0-acetyl-β-D-galactopyranoside with the immunoglobulin J539 Fab' fragment. This fact lends strength not only to our earlier postulation that D-galactopyranose-derived ligands bind only on one side of the molecule, but also to the assumption that the immunoglobulin combining-site may be on an exposed surface rather than in a deep cleft.

In addition to these results, we have already shown that, in β1,6 Galactosylgalactose the inter-saccharidic sequence from 0-1' to C-6-C-5-0-5 is important for binding to the globulin fragment. From comparison with the data on the binding of epoxypropyl β-D-galactopyranoside with IgA J539 Fab', it could be inferred that 0-4, 0-3, 0-2, and 0-1 of the reducing moiety in β1,6 Galactosylgalactose are of relatively little importance in contributing to the binding energy. A region of a molecule may not contribute to the binding, but bulky substitution could still alter the capacity of the ligand to enter a combining site if the space in that site is restricted. If, however, binding is not diminished following substitution by bulky groupings, this would indicate that the combining site may be on an exposed surface of the immunoglobulin A molecule. Thus, it became of interest to verify this for the disaccharide 3, and to prepare a derivative in which would be blocked (by bulky substituents) those oxygen atoms that we had previously postulated to be oriented away from the actual binding area of the immunoglobulin combining-site.

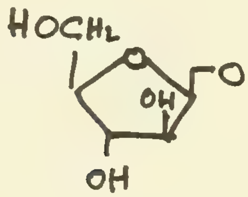
The well known syntheiss of the D-galactobiose actually employs the condensation of tetra-0-acetyl-α-D-galactopyranosyl bromide and 1,2:3,4-di-0-isopropylidene-α-D-galactopyranose, so that deacetylation of the protected disaccharide would conveniently yield a suitably protected D-galactopyranose (1), was obtained as a levorotatory, amorphous powder. It increased the tryptophanyl fluorescence of IgA J539 Fab' by 42.3%. Its n.m.r. spectrum showed two, well separated, anomeric protons and twelve methyl protons, six of which had identical chemical-shifts. The n.m.r. spectrum of vacuum-distilled

1,2:3,4-di-O-isopropylidene-α-D-galactopyranose also shows two isopropylidene methyl groups in an identical environment. Thus, the substitution of the D-galactopyranosyl group in 1,2:3,4-di-O-isopropylidene-α-D-galactose to form (1) does not cause asymmetry in the partially protected disaccharide so as to split the chemical shifts of the two identical isopropylidene methyl groups. Although several conformations can accomodate this observation, it is nevertheless true that, applied to (1), the conformation that we proposed earlier would also put the D-galactosyl group in (1) far enough away from the two isopropylidene groups (which appear to be in an almost identical environment; see below.

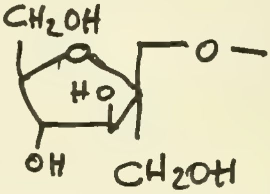


However, the major observation of interest is that the affinity constant for binding between (1) and IgA J539 Fab' is 2.6×10^4. When the free energy of binding is again calculated from this value by the equation $\Delta G=-RT$ in K_a, it is seen that the partially protected disaccharide (1) has a binding energy 8.8% higher than that of the free disaccharide; this result is not unreasonable. If substitution at O-1, O-2, O-3, and O-4 of 2 is to have little bearing on its binding with immunoglobulins it should not be supposed that 1 should of necessity bind less strongly than does galactobiose. The latter is a reducing disaccharide, and the specificity of IgA J539 is for multiple D-galactosyl residues; hence, although the (reducing) D-galactose residue will be mostly in a ring form, it is undoubtedly in equilibrium with its acyclic form to some small extent, and the latter would not bind so well to the immunoglobulin. In compound (1), the 1,2-O-isopropylidene group locks the D-galactose residue in the pyranoid form, and this would contribute to an overall higher binding-energy.

5. Homogeneous immunoglobulins with anti fructofuranan specificity. No satisfactory material has hitherto been available for affinity chromatography of these immunoglobulins. Originally it was thought that p-aminophenyl-D-arabinofuranoside could mimmick the structure of fructofuranosides (see below).



D-Arabinofuranoside



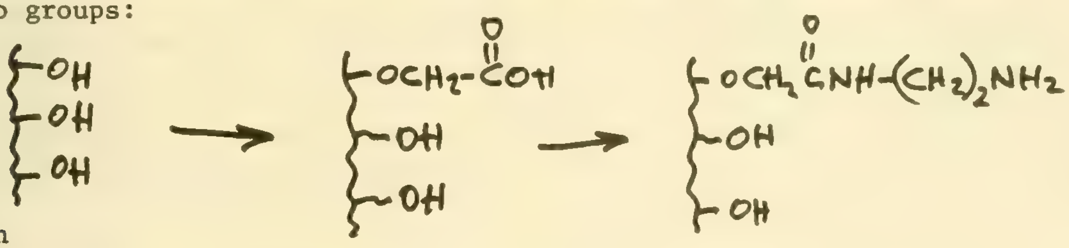
D-fructofuranoside

The arabinoside is synthetically very accessible and was prepared and coupled to bovine serum albumin (BSA). This conjugate did not precipitate with anti-fructofuranan's, however.

Subsequently the following materials were prepared: Levan (from aerobacter levanicum) or inulin were converted to their partially substituted carboxymethyl.

6. Levans from grass. Rye grass was used to obtain levan-polysaccharide for study with the homogeneous anti-fructans (grass is the only natural source of levans of low molecular weight. Crude material obtained after deproteinization of water extract from grass was further purified on two subsequent DEAE-cellulose columns. The purified material free from inorganic salts and pigments contains two levan components differing in mobility on paper electrophoresis in borate buffer. In addition a galactan component present in minute quantities was detected by means of precipitin reactions with anti-galactans.

Separation of two levan components was achieved by chromatography on DEAE Sephadex A-50 in borate buffer. Both the DEAE group, and the particle size of Sephadex proved to be essential for satisfactory separation. Both components give precipitin lines with UPC-10 immunoglobulin (anti 2→6 levan). The galactan component contaminating the main grass extract could be separated from levans by use of affinity chromatography, employing columns containing antigalactan immunoglobulin linked to Sepharose derivative. This was coupled to ethylene deamine to yield levans and inulins lightly substituted with amino groups:



Levan
or
Inulin

The resulting amino-inulin or amino-levan were coupled to CNBr-activated sepharose. These materials could be successfully employed as affinity column materials. Particularly the inulin-amino-sepharose could bind immunoglobulin with anti-fructan specificity, and these immunoglobulins could be eluted with 0-25 M sucrose solutions (with Dr. Vrana). This has opened up the study of the homogeneous fructans, which are particularly vitable for sequence studies.

7. Purification of N-acylhexosidases. 2-Acetamido-2-deoxy-D-glucono-D-galactono-, and D-mannono-1,4-lactone were each coupled to benzidine-sephacose 4B. All three materials were capable of purifying 2-acetamido-2-deoxy-β-D-hexosaminidase from epididymis. The mannono-lactone made the

best affinity column (highest purification and yield).

Significance to Biomedical Research and the Program of the Institute: The Section on Carbohydrates, with its knowledge of immunochemistry and structural carbohydrate chemistry is in a position to make a real contribution to the study of the genetics and the diversity of the immune response, employing this unique set of immunoglobulins. The specificity of antibodies and the problem of "the economy of the immune response" can be studied very well using this set of immunoglobulins.

Proposed Course. We are getting more involved in the anti fructan homogeneous immunoglobulins. This class looks very promising for a study of structure-specificity relationships of antibodies.

The study of recombinants promises answers to the questions about the idiotype of antibodies.

Keyword Descriptors: Interaction of homogeneous immunoglobulins with polysaccharide antigens.

Publications:

M. E. Jolley and C. P. J. Glaudemans: The Determination of binding constants between carbohydrate ligands and certain proteins, Carbohydrate Res., 33 (1974) 377-382.

M. E. Jolley, C. P. J. Glaudemans, S. Rudikoff and M. Potter: Structural requirements for the Binding of Derivatives of D-galactose to two Homogeneous Murine Immunoglobulins, Biochem., 13 (1974) 3179-3184.

M. Pokorny and C. P. J. Glaudemans: Purification of N-acetyl β-D-Hexosanisdase from Bull Epididymis by affinity chromatography, FEBS Letters, 50 (1975) 666-669.

C. P. J. Glaudemans, E. Zissis and M. E. Jolley: Binding Studies on a Mouse-myeloma immunoglobulin A having specificity for β-D-(1→6) linked D-galactopyranosyl residues, Carbohydrate Res., 40 (1975) 129-135.

B. N. Manjula, C. P. J. Glaudemans, E. Mushimski and M. Potter: A new Mouse myeloma Immunoglobulin A having specificity for β-D-(1→6) linked D-galactopyranosyl residues, Carbohydrate Res., 40 (1975) 137-142.

M. Pokorny, E. Zissis, H. G. Fletcher Jr. and N. Pravdic: The Inhibitory activity of 2-acetamido-2-deoxy-D-gluconolactones and their isopropylidene derivatives on 2-acetamido-2-deoxy-β-D-glucosidase, Carbohydrate Res., 37 (1974) 321-329.

H. W. Diehl, M. Pokorny, E. Zissis, R. K. Ness and H. G. Fletcher Jr: Crystalline Cellobino-1,5-lactone: its preparation and growth-inhibitory activity in the Avena coleoptile section test, Carbohydrate Res., 38 (1974) 364-368.

Project No. Z01 AM 19002-02 LC
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Preparation of 1-0-acyl glucuronic acids (of Unsaturated Acids)

Previous Serial Number: NIAMDD-LC-8

Principal Investigator: C. P. J. Glaudemans

Other Investigators: N. Roy, Council of Scientific and Industrial Research, India

Man Years:

Total:	0
Professional:	0
Other:	0

This project has been temporarily held up.

Project No. Z01 AM 19200-25 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Testing for Analgesic Activity, Dependence Liability and Narcotic Antagonism

Previous Serial Number: NIAMDD-LC #9

Principal Investigator: Everette L. May

Other Investigators: E. L. Atwell

Cooperating Units: Animal Production Section, NIH; Department of Pharmacology University of Michigan and Medical College of Virginia

Man Years:
Total: 1.5
Professional: 0.5
Other: 1.0

Project Description: This program in collaboration with the National Academy of Sciences, National Research Council, is to examine new compounds for analgesic activity (by the hot-plate and Nilsen methods), acute toxicity, properties of antagonism and abuse liability. It is of particular significance now because of an impending codeine shortage.

A total of 135 compounds from the pharmaceutical industry and universities (world-wide) and from the synthesis program of the Laboratory of Chemistry (principally Medicinal Chemistry) have been examined. Complete analgesic assays include 71 by the hot-plate, 41 by the Nilsen method. Some eighty compounds were sent to the Departments of Pharmacology, University of Michigan and the Medical College of Virginia for testing for abuse liability (both psychologic and physiologic dependence liability) and properties of narcotic antagonism. A few of these were further tested for abuse potential in man at the Addiction Research Center, Lexington. Thirty-seven toxicity experiments were performed.

Several potential codeine and morphine substitutes have emerged that appear to warrant further study. Some of these exhibit little or no abuse dependence liability in animals. Of particular interest is a (non-basic) 9-hydroxy-9-nor-hexahydro-cannabinol which shows morphine-like analgesic activity in large variety of animal tests and no physical dependence capacity in monkeys. It also has hypotensive and anticonvulsant activity.

This program will continue and the unit will also continue to advise FDA, DEA and the pharmaceutical industry on drug abuse problems.

Keyword Descriptors: Analgesic, antagonist, drug dependence, Nilsen, hot-plate, morphine, codeine.

Honors and Awards: Presented keynote speech at the 13th MIKI Meeting (Medicinal Chemistry) at the University of Iowa, March 26, 1975.

Publications: Oh-ishi, T., Jacobson, A. E., Wilson, R. S., Yeh, H. J.C. and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan. *J. Org. Chem.* 39: 1347-1349, 1974.

Wilson, R. S. and May, E. L.: 9-Nor-Δ^8-tetrahydrocannabinol, a cannabinoid of metabolic interest. *J. Med. Chem.* 17: 475-476, 1974.

Rogers, M. E. and May, E. L.: Improved syntheses and additional pharmacology of the potent analgetic; (-)-5-*m*-hydroxy-phenyl-2-methylmorphan. *J. Med. Chem.* 17: 1328-1330, 1974.

May, E. L., Bartlett, G. R., Lionetti, F. and Goldstein, G.: Chemical specifications for adenine for medical use. *Transfusion* 14: 185-187, 1974.

Inoue, H., Thyagarajan, G. and May, E. L.: Transformations in the pyridine series. *J. Heterocyclic Chem.* in press 1975.

Project No. Z01 AM 19201-01 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Preparation, biochemical and pharmacological evaluation of various, new 6,7-benzomorphans and compounds based on morphine.

Previous Serial Number: NIAMDD, LC, #12

Principal Investigator: Arthur E. Jacobson

Other Investigators: K. Rice, E. L. May, H. J.C. Yeh, R. S. Wilson and W. Klee

Cooperating Units: NIMH (W. Klee); Johns Hopkins University, School of Medicine (S. Snyder); University of Aberdeen, Scotland (H. Kosterlitz)

Man Years:
Total: 1
Professional: 1
Others: 1

Project Description: In order to find analgesics without the side-effects of the opiates, and in order to probe the nature of the narcotic receptor, several new benzomorphans, including those with unusual N-substituents, and α-chloro- β-chloro-, β-bromo- and β-iodomorphide, norheroin and 6-acetylnorheroin were prepared and pharmacologically evaluated. The usual organic, biochemical, instrumental and pharmacological techniques were employed.

The synthesized benzomorphans (e.g., 2'-hydroxy-2,5-dimethyl-9α and β-propylbenzomorphans) were morphine-like in analgesic potency and are being tested as narcotic antagonists. The analgesic potencies of all of the above-mentioned halomorphides were measured, and the toxicity of the α- and β-chloromorphides determined. The latter compound has been found in a clandestine opiate sample. A new method for obtaining various derivatives of normorphine was established; norheroin and 6-acetylnormorphine were prepared by this procedure. These compounds did not show appreciable in vivo activity, as expected. They are being tested for binding to the narcotic receptor in vitro (S. Snyder, Johns Hopkins) and on the guinea pig ileum (H. Kosterlitz, Scotland).

The synthesis of new benzomorphans, norheroin and 6-acetynormorphine coupled with the availability of testing the binding of these and the various halomorphides to narcotic receptors in vitro, has enabled us to gain greater insight into the action of analgesics in the central nervous system. Additional work in these and other areas is projected for the future.

Keyword Descriptors: Analgesics, Benzomorphans, Morphine, Halomorphides, Norheroin, 6-Acetylnormorphine, Narcotic receptors, Opiates

Publications:

Jacobson, A. E. and May, E. L.: Perspectives in the Chemistry of Narcotic Antagonists. In Braude, M. C., Harris, L. S., May, E. L., Smith, J. P. and Villarreal, J. E. (Eds.): Narcotic Antagonists. New York, Raven Press, 1973, pp. 187-189.

Katz, R., Heller, S. R., Jacobson, A. E., Rotman, A. and Creveling, C. R.: Molecular Orbital Calculations on Catecholamines; Theory and Experiment. In Bergmann, E and Pullman, B. (Eds.): Molecular and Quantum Pharmacology. Dordrecht-Holland, D. Reidel Publishing Co., 1975 in press.

Oh-ishi, T., Jacobson, A. E., Wilson, R. S., Yeh, H. J.C. and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan. J. Org. Chem. 39: 1347-1349, 1974.

Rice, K. C., Jacobson, A. E. and May, E. L.: Synthesis and analgesic activities of 2,5-dimethyl-2'-hydroxy-9α and β-propyl-6,7-benzomorphans. J. Med. Chem. in press, 1975.

Project No. Z01 19202-02 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title:
1. Synthesis and Pharmacological Evaluation of 6,7-benzomorphans
2. Synthesis and Pharmacological Evaluation of 3,6-diacetylnormorphine (norheroin) and 6-acetylnormorphine
3. Development of an Improved N-demethylation Procedure for Benzomorphans, morphine and codeine
4. Analgesic Properties of Simple Terpene Compounds
5. Study of Reactions between Glyoxal and Dimethyl-3-keto-glutarate under "Physiological" Conditions
6. Nmr study of Conformational Preferences and the nature of the N-O-bond in 1,2-oxazepins
7. Synthesis of new structural types of antiinflammatory compounds

Previous Serial Number: None

Principal Investigator: Kenner C. Rice

Other Investigators: E. L. May, A. E. Jacobson, R. Wilson, R. J. Highet, J. V. Silverton, R. E. Wasylesehen, U. Weiss, N. Sharpless and M. F. Rahman

Cooperating Units: NIMH (W. Klee); Medical College of Virginia (L. S. Harris, W. L. Dewey, B. R. Martin)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description: 1. (±)-2,5-Dimethyl-2'-hydroxy-9α and β-propyl-6,7-benzomorphan were synthesized in order to compare the experimental agonist activity with that predicted from equations previously derived (A. E. Jacobson) using a Hansch type of analysis and also because of other types of structural activity correlations which could be made. For the α-isomer, experimental and calculated activities were in good agreement. However, in the case of the β-isomer, agreement was not quite as good. This suggests that the β-isomer will show antagonist properties and this point is currently under investigation. Since it has been shown that the levo isomer of certain 6,7-benzomorphans retain essentially all the analgesic activity of the racemate, are much less toxic, and show little or no addiction liability, the optical resolution of the ± α and β isomers was carried out. As expected, the (-) α and (-) β isomers showed morphine-like, or better, mouse analgesia. Evaluation of the levo isomers for addiction liability in morphine-dependent monkeys is currently in progress. The ±-α-isomer was N-demethylated (see 3 below) and corresponding N-alkyl derivatives (C_2-C_8) were prepared for evaluation of agonist and antagonist activity. Relative to the N-methyl derivative, these compounds showed greatly reduced or no agonist activity in the mouse. Antagonist activity of these materials is currently being determined and the binding constants to narcotic receptors in vitro have been determined (W. Klee). A series of new 6,7-benzomorphans with unusual N-substituents have been prepared and are being pharmacologically evaluated.

2. A general synthesis of 3,6-diacyl and 6-acyl N-normorphines and benzomorphans was devised and used to synthesize 3,6-diacetyl-N-normorphine (norheroin) and 6-acetyl-N-normorphine. These compounds were of considerable theoretical interest since few secondary amines show good analgesic activity, presumably because they are too polar to pass through the blood-brain barrier. It was of interest to see if the acetyl groups might aid transport across this barrier. As expected, however, these two secondary amines showed only about 5% of the activity of morphine in mouse analgesia. Studies to determine the in vitro binding of these compounds to narcotic receptors are underway.

3. The N-demethylation of benzomorphans and morphine-like compounds has previously been carried out in several ways to prepare the secondary amines which are important intermediates in the synthesis of many analgesics and narcotic antagonists. In general, however, these existing methods have the disadvantages of low yields, toxicity of reagents or tedious working proceedures. Therefore, a simple, high-yield proceedure was devised which was not optimzed but which has given yields of 95%, 89% and 84% for a typical 6,7-benzomorphan, codeine, and morphine, respectively. This proceedure seems likely to be applicable for the N-demethylation of other structural types.

4. Because the structure of thujone was recently compared with that of Δ^9-THC in the literature, we have tested thujone in the hot-plate and Nilsen tests for analgesia. Thujone was essentially as active as Δ^9-THC as an analgesic and its LD_{50} was about 10 fold greater than its ED_{50}. A number of terpenes of similar structure were found to be inactive. Thujone, therefore, represents a novel, simple nonnitrogenous molecule which shows codeine-like analgesia in the mouse. Testing of its purified isomer, l-thujone and d-isothujone and some synthetic analogs is planned.

5. Previously we had shown that reaction of glyoxal and dimethyl 3-ketoglutarate under "physiological" conditions (pH 6.0, aqueous solution, 25°) produces an unsymmetrical, tetracyclic C_{25} compound in 50% isolated yield. It was also demonstrated that acid hydrolysis of this substance proceeds in good yield to give a material whose ring system corresponds to rings B, C and D of the steroids. Because of the structural similarity of this material to certain steroids of medicinal value and the ease with which this material is prepared, we have investigated certain aspects of the chemistry of this compound which may lead to compounds of medicinal value.

6. In the course of previous investigations we encountered hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one whose structure had been incorrectly assigned by early workers. This material was said to cause temporary paralysis in frogs and this property was demonstrated in mice. Because this simple, monocyclic compound showed this biological activity and also a marked conformational preference by nmr, the nature of this preference was investigated. By variable-temperature nmr, it was shown that slow conformational interconversion of the seven-membered ring and not slow inversion of the nitrogen atom was responsible for the observed conformational preference. The activation energy for conformational equilibrium was found to be 19.0 ±0.5 kcal/mol (coalescence temperature 150°). Other ^{13}C NMR experiments are in progress to study the nature of the N-O bond in this and similar compounds.

7. Synthetic studies have recently been begun with the objective of finding new, nonacidic structural types of antiinflammatory agents which lack the undesirable effects associated with the carboxylic acids. Compounds prepared will be evaluated by Hoffmann-La Roche, Nutley, N. J. and structural requirements of the active classes will be examined.

Keyword Descriptors: Nitrogenous, nonitrogenous, analgesics, N-Demethylation "Physiological" synthesis, Conformational preference, antiinflammatory.

Publications: Wasylischen, R. E., Rice, K. C. and Weiss, U: Nuclear magnetic resonance evidence for high barrier of conformational inversion in hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one. Canad. J. Chem. 53: 414-418, 1975.

Rice, K. C., Jacobson, A. E. and May, E. L.: Synthesis and analgesic activities of 2,5-dimethyl-2'-hydroxy-9α- and β-propyl-6,7-benzomorphans. J. Med. Chem. in press 1975.

Rice, K. C.: An improved proceedure for the N-demethylation of 6,7-benzomorphans, morphine and codeine. J. Org. Chem. in press, 1975.

Project No. Z01 AM 19203-02 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title:
1. Mechanism of action of Δ^8- and Δ^9-tetrahydrocannabinol (THC) from marihuana.
2. Analgesic properties of the THC's and their metabolites.
3. Analgesic and other properties of synthetic cannabinoid-like compounds.
4. Interaction of opiate analgesics and prostaglandins in the guinea pig ileum.
5. Structure determination, receptor binding, and biological activity of halomorphides.
6. Analgesic properties of some terpenoid compounds.

Previous Serial Number: NIAMDD, LC, #11

Principal Investigator: Raymond S. Wilson

Other Investigators: E. L. May, A. E. Jacobson, H. J.C. Yeh, K. Rice, W. Klee, L. S. Harris, W. L. Dewey, B. R. Martin, S. Turkanis

Cooperating Units: ADAMHA (W. Klee); Medical College of Virginia (L. S. Harris, W. L. Dewey, B. R. Martin); University of Utah College of Medicine (S. Turkanis).

Man Years:
Total: 2
Professional: 1
Other: 1

Project Descriptions: 1) An improved synthesis of 9-nor-Δ^9-THC was devised and this compound was tested. It was similar both quantitatively and qualitatively to Δ^9-THC in dog behavioral and cardiovascular tests and in the mouse activity cage test. These results suggest that the 11-hydroxy metabolite is not needed for these biological activities of Δ^9-THC, similar to our previous finding for Δ^8-THC. These synthetic compounds will be used to examine the role of metabolic 11-hydroxylation in other cannabinoid activities. The results of this study are useful in determining now the cannobinoids exert their effects and in defining structure activity relationships of the cannabinoids.

2) The THC's, a large number of known metabolites including mono- and dihydroxy and carboxy compounds, and compounds lacking the 11-methyl were

examined in the hot-plate and Nilsen analgesia tests. The results suggested that most or all of the analgesia was due to the 11-hydroxy metabolites, but not due to products of further metabolism. These results will help understand how the THC's produce analgesia and be useful in the design of synthetic cannabinoid-like analgesics. This also emphasizes the importance of metabolism in the action of some drugs.

3) 9-nor-9β-Hydroxyhexahydrocannabinol (9β-OH-HHC) was previously found to be of morphine-like potency in the hot-plate test for analgesia whereas its 9-αhydroxy isomer (9α-OH-HHC) was essentially inactive. 9β-OH-HHC has now been found to be essentially morphine-like in the Nilsen, writhing, tail-flick, and bradykinnin tests for analgesia as well. At doses producing CNS effects, 9β-OH-HHC failed to substitute for morphine in single dose suppression of morphine abstinence tests in monkeys. Both 9α- and 9β-OH-HHC displayed CNS depressent effects, hypotensive effects, and anticonvulsant activity in preliminary tests. Four other synthetic compounds with 9-hydroxy groups were prepared and were less active as analgesics than 9β-OH-HHC but all had CNS effects. This new class of synthetic compounds represents a a possibly non-addicting class of potent analgesics, hypotensives, anticonvulsants, and CNS depressents. Anti-inflammatory activity will also be examined.

4) A paper has appeared describing the morphine antagonism of PGE, stimulation of adenyl cyclase in rat brain homogenate and it was suggested that this may be the mechanism by which morphine acts. We examined both homogenates and slices of guinea pig ileum, a classic model for evaluating opiates, and found that none of a series of opiate agonists or antagonists affected the PGE stimulation of adenyl cyclase. This suggests that either morphine acts by different mechanisms in the brain and in the ileum or else its effect in the brain is not actually due to inhibition of the PGE-stimulated adenyl cyclase.

5) Recently β-chloromorphide was identified as a constituent of an illicit opium sample in a U. S. customs lab. We determined the structure of both α- and β-chloromorphide (previously not known with certainty) by nmr analysis along with some bromo- and iodomorphides. Toxicity, analgesic potencies, and *in vitro* binding to an opiate receptor were determined for all of the compounds. Since these compounds are faily reactive allylic halides, they were possible opiate receptor alkylating agents. However, none of the compounds appeared to be irreversible binders to the receptor.

6) Because the structure of thujone was recently compared with that of Δ^9-THC in the literature, we have tested thujone in the hot-plate and Nilsen tests for analgesia where we had previously tested Δ^9-THC and its analogs. Thujone was about as active as Δ^9-THC as an analgesic and its LD_{50} was about ten-fold greater than its ED_{50}. A number of terpenoids of similar structure were tested and found to be inactive. This represents a novel, simple, non-nitrogenous molecule which is an analgesic. Testing of its purified isomers, *l*-thujone and *d*-isothujone, and some synthetic analogs is planned.

Keyword Descriptions: Marihuana, tetrahydrocannabinol, tetrahydrocannabinol-metabolism, analgesia, opiate receptor, guinea pig ileum, morphine, thujone.

PUBLICATIONS: Oh-ishi, T., Jacobsen, A. E., Wilson, R. S., Yeh, H.J.C., and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan. J. Org. Chem. 39: 1347-1349, 1974.

Wilson, R. S., Rogers, M. E., Pert, C. B. and Snyder, S. H.: Homologous N-alkylnorketobemidones. Correlation of receptor binding with analgesic potency. J. Med. Chem. 18: 240-242, 1975.

Wilson, R. S. And May, E. L.: Analgesic properties of the tetrahydrocannabinols, their metabolites, and analogs. J. Med. Chem. in press, 1975.

Martin, B. R., Dewey, W. L., Harris, L. S., Beckner, J., Wilson, R. S. and May, E. L.: Marihuana-like activity of new synthetic tetrahydrocannabinols. Pharmacol. Biochem. Behavior in press, 1975.

Wilson, R. S.and May, E. L.: The role of 11-hydroxylation in THC activity. In Braude, M. and Szara, S. (Eds.): Pharmacology of Marihuana. New York, Raven Press, 1975, in press.

Project No. Z01 AM 19204-03 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Titles:
1) Synthesis, analgetic evaluation, and "receptor affinities" of N-alkyl analogs of 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine
2) Synthesis of 3a-aryloctahydroindoles as adrenergic agents
3) Nature of histamine receptors in guinea pig brain
4) Development of cAMP phosphodiesterase inhibitors

Previous Serial Number: NIAMDD, LC, #13

Principal Investigator: Michael E. Rogers

Other Investigators: Everette L. May, John W. Daly, C.R. Creveling, R.K. Dismukes, NIAMDD, LC; Werner Klee, NIMH, ADAMHA

Cooperating Units: Microanalytical Services and Instrumentation Section

Man Years:

Total: 1
Professional: 1
Others: 0

Project Description: 1) N-Alkyl (C_2-C_7) analogs of 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine were prepared as narcotic agonists-antagonists; mouse hot-plate analgetic potencies, antagonist data in the morphine-dependent monkey and receptor binding affinities in rat brain homogenates were obtained for the new compounds. Although the compounds were devoid of typical antagonist activity and lacked sufficient analgetic potency to be of therapeutic interest, a significant correlation was observed between analgetic potencies and binding affinities to brain homogenates providing additional evidence that these binding sites are involved in the narcotic analgesia. Work on this project has been terminated.

2) As semi-rigid phenethylamines, cis-3a-(m-methoxyphenyl) and cis-3a-(m-hydroxyphenyl)-octohydroindole were prepared and examined for effect on uptake and release of norepinephrine in mouse heart. Both compounds strongly inhibited uptake and neither caused release. Whole animal studies have been initiated. Additional analogs, especially the trans isomers, are being synthesized in an effort to delimit structural and stereochemical requirements for the catecholamine uptake system.

3) The nature of the histamine receptors involved in the stimulation of formation of cAMP in guinea pig brain slices was investigated using selective H_1 and H_2 agonists and antagonists. It was shown that stimulation was the greatest in the cortex and hippocampus, that the receptors in the cortex were predominately of the H_1 type and those in the hippocampus were a fairly even mixture of H_1 and H_2. Furthermore, it was shown that the synergism observed between adenosine and histamine in the production of cAMP was mediated primarily by the H_1 histamine receptors and the affinity of these receptors for histamine is increased in the presence adenosine. Additional studies are being undertaken to determine if the cAMP levels observed were influenced by the action of the H_1 and H_2 agonists used on cAMP phosphodiesterase.

4) An effort has been initiated to develop selective inhibitors of cAMP phosphodiesterase. Such compounds could have considerable importance as therapeutic agents and pharmacological tools. Assay for PDE activity has been set up which measures labelled 5'-AMP produced after purification by ion-exchange chromatography. Initial studies are being conducted to determine if sulfhydryl groups are in or near the active site of the enzyme. If this is the case, compounds will be designed and synthesized which will have affinity for the active site and contain moieties that would react with sulfhydryl groups to irreversibly inhibit the enzyme.

Keyword Descriptors: Benzazocines, Receptors, Octahydroindoles, Adrenergics, Histamine, cAMP, Inhibitors, Phosphodiesterase, Binding, Analgetic.

Publications: Rogers, M. E. and May, E. L.: Improved synthesis and additional pharmacology of the potent analgetic (-)-5-m-hydroxyphenyl-2-methylmorphan. J. Med. Chem. 17: 1328-1330, 1974.

Wilson, R. S., Rogers, M. E., Pert, C. B., and Snyder, S. H.: Homologous n-alkylnorketobemidones. Correlation of receptor binding with analgesic potency. J. Med. Chem. 18: 240-242, 1975

Rogers, M., Dismukes, K., and Daly, J. W.: Histamine-elicited accumulations of cyclic adenosine 3',5'-monophosphate in guinea pig brain slices. J. Neurochem., in press.

Project No. Z01 AM 19205-02 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Synthesis of 9-methyl-6,7-benzomorphans and their derivatives.

Previous Serial Number: NIAMDD, LC, #16

Principal Investigators: Hirozumi Inoue and E. L. May

Other Investigators: None

Cooperating Units: Pharmacology Unit and MIcroanalytical Services and Instrumentation Section

Man Years:
Total: 1
Professional : 1
Other: 0

Project Description: a) 2'-Hydroxy-2,9α-dimethyl-6,7-benzomorphan was synthesized in 1.9% yield from *m*-methoxyphenylacetone via the 3,4-*trans* isomer of 4-(2-dimethylaminoethyl)-3-methyl-6-methoxy-3,4-dihydro-1(2H)-naphthalenone and evaluated for analgesic activity.

Intramolecular aminomercuraton of 1-(2-monomethylaminoethyl)-2-methyl-7-methoxy-1,2-dihydronaphthalene which was prepared by a four-step procedure from *trans*-4-(2-dimethylaminoethyl)-3-methyl-6-methoxy-1-tetralone was the key step of this route.

After this, the methods of the 2'-hydroxy-2,9βb-dimethyl series were applicable for obtaining 2-8α-dihydroxy-2,9α-dimethyl-6,7-benzomorphan. (see 1973-1974 project report)

(b) 2,9α-Dimethyl-6,7-benzomorphan was synthesized from phenylalanine *via* 2-benzylmethylamino-6-methoxy-3-methyl-1-phenyl-3-hexene in low yield.

Key steps of this route were dehydration of 2-benzylmethylamino-6-methoxy-3-methyl-1-phenyl-3-hexanol and then cyclization to the 1,2,3,4-tetrahydronaphthalene derivative. Standard methods were applicable for other steps.

The 2,9α-dimethyl isomer was not obtained by this new route.

The other route via cyclization of 1,3-dimethyl-2-hydroxycarbonyl-4-phenylpiperidine was unsuccessful. Both the α- (as potent as pethidine as an analgesic) and β- (as strong as morphine) isomers act as antagonists in morphine-dependent monkeys and are devoid of physical dependence capacity.

c) 2,9α-Dimethyl-6,7-benzomorphan and N-derivatives of the 2,9-dimethyl-6,7-benzomorphan series lacking an alkyl substituent at C-5 must be synthesized and evaluated.

Keyword Descriptors: 2'-Hydroxy-2,9α-dimethyl-6,7-benzomorphan, intramolecular, aminomercuration, analgesics.

Publications: Inoue, H., Thyagarajan, G., and May, E. L.: Transformations in the pyridine series. An improved preparation of 3-methyl-4-phenylpyridine. J. Heterocyclic Chemistry in press 1975.

Project No. Z01 AM 19207-02 LC
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of Norepinephrine in the Perception of Pain

Previous Serial Number: NIAMDD, LC, #15

Principal Investigator: Ludmilla Syrotenko-Getsiv

Other Investigatiors: None

Cooperating Units: None

Man Years:
Total: 1.6
Professional: 1.6
Other: 0

Project Description: To investigate the role of catecholamines (CA) in the phenomenon of nociception, obejctives of the Research Proposal, including bibliography, detailed in the Project Report of April 12, 1974. The specific objective for Phase I of the Project, the subject of this report, was to elucidate the role of norepinephrine (NE) in the conscious component of the process of pain perception.

Nilsen method, which utilizes electrical stimulus as nociceptive agent and quantitative vocalization as the end-point, was employed throughout the study. All work has been done in vivo, using a mouse as an experimental model. The work comprised 10 studies, including the control background, involving some 240 mice.

Endogenous levels of CA, particularly those of NE, were modified by means of selective enzyme inhibition, standard pharmacologic intervention and without recourse to chemical intervention.

The endogenous levels of NE were decreased, up to 38% of control, by inhibition of dopamine-β-hydroxylase (DBH), enzyme which catalyzes the last step of NE synthesis:-β-hydroxylation of dopamine (DA). The inhibitors used included thiourea (U-14,624 a 1-thenyl-3(2-thiazolyl)2-thiourea, 200 mg/kg) and dithiocarbamate (dimethyl-dithiocarbamic acid, $Na \cdot 2H_2O$, 500 mg/kg.). The choice of these inhibitors, in addition to NE synthesis inhibition, had also provided data concerning the role of DA.

Endogenous NE levels were increased by inhibition of its metabolism by catechol-O-methyltransferase (COMT), using pyrogallol (200 mg/kg) as an inhibitor, and by injection, i.v. and s.c., of NE solutions of varying concentrations. Reserpine (0.7 mg/ml. drinking H_2O) and 6-hydroxydopamine (6-OHDA), (100 mg. and 200 mg/kg) were used to deplete CA stores while their elevation was attempted with dietary L-Tyrosine (7.5 mg and 10 mg/ml. drinking H_2O).

In the final, definitive, set of experiments, the endogenous levels of NE were regulated by means of quantitatively graded total doses of electrical stimulation.

In addition to the experimental data, the continuous, dated and timed, records of the following parameters were kept: weight, water and food intake, ambient temperature, weather and the record of reproducibility of the magnitude of voltage delivered by the stimulator employed in the study.

The experimental results of the study demonstrate the existence of a heretofore unknown quantitative relationship, a directly proportional one, between the endogenous concentrations of NE and the nociceptive vocalization threshold in mice, namely: the increase of the endogenous NE above the control level results in a corresponding increase in the vocalization threshold, while a decrease in endogenous NE decreases the vocalization threshold. This finding has been verified to be a consistent one by studies intentionally based on varying experimental rationales. Although the evidence regarding the possible contributory role of DA is not yet complete, there is a strong indication that DA as such is not directly involved in the above relationship.

Other positive findings indicate the metabolic nature of the observed diminished average weight gain in mice subjected to repeated electrical stimulation, involving also, as yet, not sufficiently quantitated changes in dietary habits. The above is believed to be a consequence of depressed levels of endogenous NE; however, further data are needed to support this view.

Vocalization in mice, as a reaction to a painful stimulus, may be considered to represent a conscious response component in the process of perception of pain and, as such, to serve as an approximation of the cognitive perception of pain in humans. Demonstration of the direct reletionship between the endogenous levels of NE and the acutenes of pain perception - introduces a new basic factor for consideration in elucidation of the phenomena of nociception and analgesia, in both - theoretical and practical aspects. Recognition of such relationship is important in the search for means of alleviation of pain and provides an added insight into the basic mechanism of nociception.

Some purely practical benefits include: (1) Demonstration of applicability of the Nilsen method for: (a) preselection of populations of mice with either low or high endogenous NE; (b) monitoring of intentional modifications of such levels in chronic studies; (c) as preliminary indicator in some enzyme-induction studies. (2) Direction of attention toward the need to consider the role of endogenous NE in the evaluation of experimental data obtained with the Nilsen method, or other methods involving electrical stimulation.

Since my appointment terminates in September, 1975, no discussion of proposals for the future is included; the remaining time is to be spent on completion of work in progress.

Keyword Descriptors: Vocalization threshold; mouse; enzyme inhibition; dopamine--hydroxylase (DBH); catecholamines; catechol-O-methyltransferase (COMT).

Publications: None.

Project No. Z01 AM 19400-10 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Batrachotoxin, Pumiliotoxin, Histrionicotoxin and Other Physiologically Active Compounds from Neotropical Frogs.

Previous Serial Number: NIAMDD-LC-23

Principal Investigator: Bernhard Witkop

Other Investigators: J. Daly, G. Brown, and W. Kissing, E. Gossinger

Cooperating Units:
Charles W. Myers, American Museum of Natural History, New York, N. Y.
E. X. Albuquerque, Dept. of Pharmacology, University of New York at Buffalo, N. Y.
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.
G. Habermehl, Technische Hochschule, Darmstadt, Germany
T. Tokuyama, Osaka City University, Osaka, Japan
E. J. Corey, Harvard University
Y. Kishi, Harvard University
H. Wehrli, ETH, Zurich, Switzerland
E. Bartels de Bernal, Universidad de Valle, Cali, Columbia
M. Mensah-Dwumah-Guest Worker, Howard University, Washington, D. C.

Man Years:

Total: 1
Professional: .5
Other: .5

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, tetrodotoxin, histrionicotoxin and related substances. To isolate and elucidate the structures of other pharmacodynamically active substances found in skin extracts of neotropical frogs. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, high pressure liquid, column and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, x-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthetic pathways. Biological activity has been investigated with both isolated enzyme systems and in intact cells.

Major Findings: Additional quantities of histrionicotoxins and bactrachotoxins have been isolated for further investigation of their unique pharmacological properties. Radioactive batrachotoxin of higher specific activity has been prepared and appears to bind to specific sites in membrane fragments from mammalian muscle.

Investigations of alkaloids from skin extracts of fifteen different species of dendrobatid frogs by a combination of gas chromatography-mass spectrometry (electron impact and chemical ionization techniques), coupled with isolation by thin-layer or column chromatography and high resolution mass spectrometric analysis of the isolated compounds has demonstrated the occurence of 85 different alkaloids to which many can now be assigned probable structures. Many of these compounds have interesting pharmacological activity. For example, a trace alkaloid present in at least three species has been found to elicit a Straub tail reaction in mice and to be a more potent analgesic than morphine. Another causes a sustained penile erection after subcutaneous injecton in mice. Efforts to isolate sufficient quanitites of these two alkaloids for structure determination and further pharmacological investigation are in progress. Extracts from a variety of other frogs are under investigation. A water-soluble toxic substance has been detected in extracts from colostethus inguinalis and a chloroform-soluble neutral toxin in extracts from atelopus ignescens. Alkaloids have been detected only in extracts from dendrobatid frogs. A new dihydrohistrionicotoxin containing an acetylenic moiety in the five carbon side chain has been isolated and is under pharmacological investigation.

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Because of their remarkable effects on ion conductances, histrionicotoxin and various analogs provide unique tools for the study of the acetylcholine modulated ion channels in electrogenic membrane. Other compounds from frog extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dehydrobufotenine is relevant to fundamental aspects of neurochemistry.

Proposed Course of Project: The structures of various alkaloids from frogs of the genera *Dendrobates*, *Colosthetus* and *Phyllobates* will be elucidated. The pharmacological activity of batrachotoxin and histrionicotoxin analogs will be studied in detail. Radioactive batrachotoxin, tetrodotoxin and histrionicotoxin will be prepared and used to study their ultrastructural site of action. Further isolations of batrachotoxins, histrionicotoxins, and pumiliotoxins will be carried out to provide material for chemical and pharmacological study. Pharmacologically active substances will be sought for in extracts from other families of tropical frogs.

Keyword Descriptors: Neurotoxins, mass spectrometry, membranes

Publications:

Warnick, J. E., Albuquerque, E. X., Onur, R., Jansson, S.-E., Daly, J. W., Tokuyama, T., and Witkop, B.: The pharmacology of bactrachotoxin. VII. Structure-activity relationships and the effects of pH. *J. Pharmacol. Expt. Therap*. In press.

Lapa, A. J., Albuquerque, E. X., Sarvey, J. M., Daly, J. W., and Witkop, B.: Effects of histrionicotoxin on the chemosensitive and electrical properties of skeletal muscle. *Exptl. Neurol*. In press.

Project No. Z01 AM 19401-10 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Photocyclizations

Previous Serial Number: NIAMDD-LC-22

Principal Investigator: Bernhard Witkop

Other Investigators: None

Cooperating Units: O. Yonemitsu, Y. Kanaoka and T. Iwakuma, University of Hokkaido, Sapporo, Japan
N. Kanamaru and K. Kimura, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan
I. L. Karle, U. S. Naval Research Lab., Washington, D.C.

Man Years:

Total: 1.0
Professional: .5
Others: .5

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods were applied only recently to this problem. Photochemical methods have been elaborated which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines and certain of their derivatives therefore have been investigated.

Methods Employed: Ultraviolet irradiation, flash photolysis thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: Stereospecific Acid-Catalyzed Rearrangement of 1,6-Dimethylpentacyclo[6,4,0,$0^{2,7}$,-$0^{3,10}$,$0^{6,9}$]dodeca-5,12-dione to a Bisnordiadamantane - On treatment with trifuoroacetic acid at room temperature cage compounds, such as pentacyclo[6,4,0,$0^{2,7}$,$0^{3,10}$,$0^{6,9}$] dodeca-5,12-diones having a methyl group at C-2 synthesized photochemically from Diels-Alder dimers of cyclohexa-2,4-dienones, reverted easily to the dimers in high yield. Stabilization of a carbonium ion at C-2 by a methyl group was assumed to be the most important requirement for this acid-catalyzed reversion.

A cage compound with a methyl group at C-1, on acid treatment would be expected to release most of the strain of the bicyclo[2,2,0]hexane system in a different way:

When such a methyl homolog was heated under reflux in trifluoroacetic acid for 15 min, or in benzene with p-TsOH for 45 min, the isomeric rearrangement product was isolated in almost quantitative yield.

Protonation of one of the carbonyl groups in the methyl homolog causes the formation of the methyl-stabilized carbonium cation at C-1 through rearrangement of either bond a or b, followed by another set of two-fold Wagner-Merrwein rearrangements to yield a less strained cage compound. Thus treatment of 1,6-dimethylpentacyclo[6,4,0,$0^{2,7}$,$0^{3,10}$,$0^{6,9}$]dodeca-5,12-dione with trifluoroacetic acid or p-TsOH gave bisnordiadamantane, 1,5,6,6-12,12-hexamethylpentacyclo[6,3,1,$0^{2,7}$,$0^{3,10}$,$0^{5,9}$]dodecane-4,11-dione, through two sets of stereospecific two-fold Wagner-Meerwein rearrangements in almost quantitative yield.

Significance to Biomedical Research and the Program of the Institute: New approaches to novel heterocyclic systems of potential CNS activity.

Proposed Course: Extension into homo- and heterocyclic systems relating to pharmacodynamic amines and to potential antiviral cage compounds.

Keyword Descriptors: Photochemical approaches to novel heterocyclic systems from N-chloroacetyl derivatives of pharmacodynamic amines and to novel cage and adamantane derivatives with potential antiviral activity.

Honors and Awards: None

Publications:

Kanaoka, Y., Koyama, K., Flippen, J. L., Karle, I. L., Witkop, B.: Photocyclizations of N-Alicyclic Phthalimides. Synthesis of Multicyclic Benzazepine Systems, JACS, in press.

Iwakuma, T., Nakai, H., Yonemitsu, O., Witkop, B.: Photocyclizations of N-Chloroacetyltyramine. I. Formation of Novel Dimeric Cage Compounds. JACS 96, 2564-2569 (1974).

Project No. Z01 AM 19402-02 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Synthesis, Chemistry and Biological Activities of Nucleic Acids and Their Constituents. Interferon Induction By Synthetic Polynucleotides

Previous Serial Number: Consolidation of NIAMDD-LC-17, NIAMDD-LC-18, NIAMDD-LC-21, NIAMDD-LC-24, NIAMDD-LC-25

Principal Investigator: Paul F. Torrence

Other Investigators: Bernhard Witkop, James A. Waters, Bharat Bhooshan (since March 1975)

Cooperating Units: Erik De Clercq, Rega Institute, Leuven, Belgium; David Stollar, Tufts University, Boston, Mass.; Albert Bobst, University of Cincinnati, Ohio; Jan Vilcek, New York University; Struther Arnott, Purdue University

Man Years

Total:	1.4
Professional:	1.2
Other:	0.2

Project Description:

Objectives: To synthesize modified nucleosides, nucleotides and polynucleotides. To characterize such materials by physicochemical means and then investigate the relationship of molecular structure and conformation to certain biochemical and biological activities of nucleic acids. The chief goal of this project is to provide 1) nucleic acid analogs which may be useful in understanding the biochemistry of cytolytic and oncogenic viruses and/or 2) the prophylaxis or therapy of viral diseases. In this latter area, considerable emphasis is currently being placed on the development of an ideal synthetic interferon inducer which may be used in the production of exogenous or endogenous human interferon.

Methods Employed: Thin-layer, paper, ion-exchange, gel and other types of column chromatography, liquid scintillation counting, ultracentrifugation, ultraviolet, visible, infrared and nuclear magnetic resonance, spectroscopy, mass spectroscopy, electron spin resonance, circular dichroism, tissue culture.

Major Findings:

1. Interferon Induction: Tool for Establishing Interactions Among Polynucleotides.

Interferon induction in primary rabbit kidney cells, "superinduced" with inhibitors (actinomycin D and cycloheximide), can be effectively employed to monitor polynucleotide triplex formation and polynucleotide displacement reactions. This is the first biological system which has been demonstrated to possess the needed specificity to discriminate unambiguously between single-, double- and triple-stranded RNA, between DNA and RNA, and even between different double-stranded RNA's. The following represents a small sampling of the polynucleotide interactions demonstrated with this interferon system in conjunction with classical biochemical and physical techniques (such as ribonuclease sensitivity, sucrose gradient ultracentrifugation, ultraviolet mixing curves and melting profiles).

a) poly(A) · poly(U) + poly(I)----poly(A) · poly(U) · poly(I)

b) poly(A) · 2poly(I) + poly(U)----poly(A) · poly(U) · poly(I) + poly(I)

c) poly(A) · 2poly(I) + 2poly(U)----poly(A) · 2poly(U) + 2poly(I)

d) poly(X) + poly(A) · poly(U)----poly(A) · poly(U) · poly(X)

e) poly(A) + poly(U) · poly(X)----poly(A) · poly(U) · poly(X)

f) poly(U) + poly(A) · poly(X)----poly(A) · poly(U) · poly(X)

g) poly(U) + poly(A) · poly(br^{5}U)----poly(A) · poly(br^{5}U) + poly(U)

h) poly(A) · 2poly(I) + 2poly(br^{5}C)----2poly(I) · poly(br^{5}C) + poly(A)

i) poly(c^{7}A) · poly(rT) + poly(A)----poly(A) · poly(rT) + poly(c^{7}A)

[poly(c^{7}A)≡poly(7-deazaadenylic acid)]

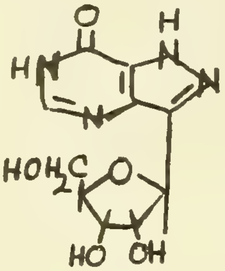
j) poly(L) · poly(br^{5}C) + poly(c^{7}I)----poly(c^{7}I) · poly(br^{5}C) + poly(L)

[poly(L)≡polylaurusin; poly(c^{7}I)≡poly(7-deazainosinic acid]

Such studies have, for instance, demonstrated the occurrence of 19 previously unknown displacement reactions and have confirmed the generalization that such displacements proceed in the direction of the helix with the higher Tm; furthermore, we have shown that such displacements also proceed to give the helix with the greater interferon inducing capacity and that all such reactions occur at a temperature far below the Tm of the least stable complex. The occurrence of reactions a, b and d-f above documents for the first time polynucleotide triplexes containing three different heterocyclic bases. Such unusual triplexes are of fundamental interest for the understanding of the potential role of nucleic acid triplexes in nature; for instance, the folded chromosome of *E.coli* and the regulation of the expression of the gene in eukaryotic systems.

2. Polynucleotide structural requirements for the induction of Interferon.

Through the use of a number of polynucleotides modified in either the purine or pyrimidine ring we have established that not only is the induction process remarkably sensitive to the nature of the ribose phosphate backbone of the double-helical complex, but is also sensitive to the nature of the bases in the interior of the double-helix; for instance, polynucleotides based on poly(L) (polylaurusin)



fail to induce interferon even at concentrations 10,000 x greater than that effective in the case of poly(I) · poly(C). We have additionally found that the recognition of modified polynucleotides by antisera to double-stranded RNA (e.g. poly(A) · poly(U)) is dramatically dependent on a) the nature of the ribose phosphate backbone and b) the bases on the interior of the double-helix. These results suggest that double-helix conformation determines the interferon inducing ability of a RNA molecule by defining the interaction of the RNA with the cellular receptor site for nucleic acid interferon inducers.

It has also been demonstrated that in both primary rabbit kidney cells and in human skin fibroblast cells, the interferon inducing capacity of both poly(I) · poly(C) and poly(c^{7}I) · poly(C) is surpassed by their 5-bromosubstituted analogs, poly(I) · poly(br^{5}C) and poly(c^{7}I) · poly(br^{5}C). The results obtained in human diploid cells currently indicate that for human interferon production purposes in cell culture, poly(I) · poly(C) may be advantageously replaced by its analog, poly(I) · poly(br^{5}C), at least at lower dosage regimens.

3. Thiocyanato Pyrimidine Nucleosides as Antiviral Agents.

Our earlier studies showed that 5-thiocyanato-pyrimidine nucleosides are useful intermediates for the facile preparation of the biologically active 5-mercapto-pyrimidine nucleosides. We have now found that such 5-thiocyanato-pyrimidine nucleosides are themselves effective antiviral agents. One such compound, 5-thiocyanato-2'-deoxyuridine, is active at 4 μg/ml (minimum inhibitory concentration) in inhibiting the cytopathogenicity of either vaccinia virus or herpes simplex virus in primary rabbit kidney cells. In one animal model, vaccinia virus-induced tail lesions in mice, 5-thiocyanato-2'-deoxyuridine (5-SCNdU) proves nearly as efficacious as 5-iodo-2'-deoxyuridine. Studies are currently in progress with this nucleoside in herpes simplex induced encephalitis in mice. In tissue culture, toxic effects with (5-SCNdU) became barely noticeable at 200 µg/ml and at this same high concentration (in non-infected cells) a seemingly paradoxical effect is witnessed: 5-SCN-2'dU stimulates (10-30X) the incorporation of labeled thymidine into DNA. Further studies are envisioned to attempt to unravel this apparently novel mechanism of antiviral activity.

4. Inhibition of Oncornavirus Functions by Polynucleotides.

The 2'-azido analog of poly(U), poly(dUz)[poly(2'-azido-2'-deoxyuridylic acid], first synthesized in this laboratory, was found to inhibit the RNA-directed DNA polymerase (reverse transcriptase) activity of murine leukemia (Moloney, Rauscher) and sarcoma (Moloney) virus, and feline leukemia (Theilen) and sarcoma (Gardner) virus, while under the same conditions the unsubstituted parent compounds failed to do so. In addition, poly(dUz) inhibited the replication of exogenous murine sarcoma virus (Moloney) in nontransformed cells (as assessed by an infectious center assay), but poly(dUz) failed to suppress the formation of endogenous sarcoma and leukemia viruses in transformed cell lines. In these same cell lines, poly(dUz) failed to inhibit the multiplication of vesicular stomatitis virus. These data add further strength to the contention that reverse transcriptase is necessary for the productive infection and transformation of normal cells by oncornaviruses but is not essential for the maintenance of this transformed state and the continuous production of new virus particles by these transformed cells.

Significance to Biomedical Research and the Program of the Institute: Studies described herein and those in progress are of interest for the following reasons.

1) Modified nucleosides can aid in understanding the biochemistry of viruses; in addition, they are of immediate interest as agents active against viral and neoplastic disease and, in some instances, as local agents in rheumatoid arthritis.

2) Modified polynucleotides aid in the understanding of protein-nucleic acid interactions and nucleic acid-nucleic acid interactions. These considerations are fundamental for a complete knowledge of cellular biochemistry including on the one hand the regulation of expression of genetic information in eukaryotic cells and, at the more practical level, the induction of interferon and the design of a clinically useful interferon inducer.

Proposed Course:

1. Poly(2'-azido-2'-deoxyuridylic acid) will be examined for its ability to inhibit tumor development and death by oncogenic viruses in mice.

2. Investigations into the chemistry of novel nucleic acid triplexes will be continued with special emphasis on DNA, and RNA-DNA mixed triplexes and those containing guanosine residues. The immunochemistry of triple-helical nucleic acids will be investigated.

3. Studies on the biochemical mode of action of 5-thiocyanato-2'-deoxyuridine will be continued and its efficacy in model animal infections will be determined.

4. A number of previously synthesized polynucleotides will be prepared to be evaluated as interferon inducers.

5. Approaches to the synthesis of novel nucleosides and polynucleotides will be explored.

Keyword Descriptors: Interferon, antiviral agents, polynucleotides, double-stranded RNA, antibodies, reverse transcriptase, recognition of nucleic acids, nucleosides, nucleotides, triple-helical nucleic acids.

Honors and Awards: None

Publications:

Torrence, P. F., De Clercq, E., Waters, J. A. and Witkop, B.: A Potent Interferon Inducer Derived From Poly(7-deazainosinic acid), Biochemistry 13: 4400-4408, 1974.

De Clercq, E., Torrence, P. F., Witkop, B., Stewart II, W. E. and De Somer, P.: Interferon Induction: Tool for Establishing Interactions Among Homopolyribonucleotides, Science 186: 835-837, 1974.

Torrence, P. F., De Clercq, E., Waters, J. A. and Witkop, B.: Failure of Duplexes Based on Polylaurusin [Poly(L), "Polyformycin B"] To Induce Interferon, Biochem. Biophys. Res. Comm. 62: 658-664, 1975.

De Clercq, E., Billiau, A., Hobbs, J., Torrence, P. F. and Witkop, B.: Inhibition of Oncornavirus Functions by 2'-Azido Polynucleotides, Proc. Nat. Acad. Sci. USA 72: 284-288, 1975.

De Clercq, E., Torrence, P. F., De Somer, P. and Witkop, B: Biological, Biochemical and Physicochemical Evidence for the Existence of Polyadenylic • Polyuridylic • Polyinosinic Acid Triplex, J. Biol. Chem. 250: 2521-2531, 1975.

Torrence, P. F. and Witkop, B.: Polynucleotide Duplexes Based On Poly(7-deazaadenylic acid), Biochim. Biophys. Acta: in press.

Project No. Z01 AM 19403-02 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Synthesis and Elucidation of Natural Products

Previous Serial Number: NIAMDD-LC-29

Prinicipal Investigator: Thomas F. Spande

Other Investigators: Edda Gössinger

Cooperating Units: None

Man Years

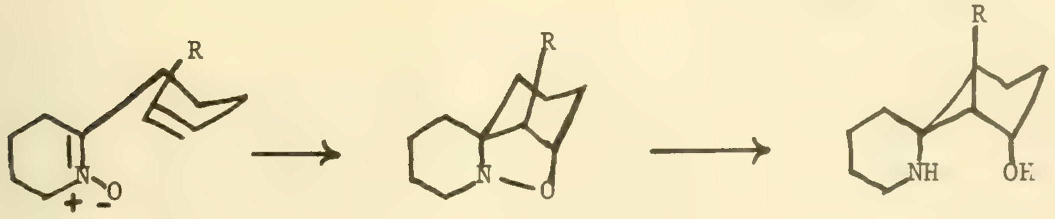
Total:	1.0
Professional:	0.5
Other:	0

Project Description:

Objectives: To synthesize analogs of the potent natural frog toxin, histrionicotoxin.

Methods Employed: IR and NMR spectroscopy, mass spectrometry, thin layer and column chromatography.

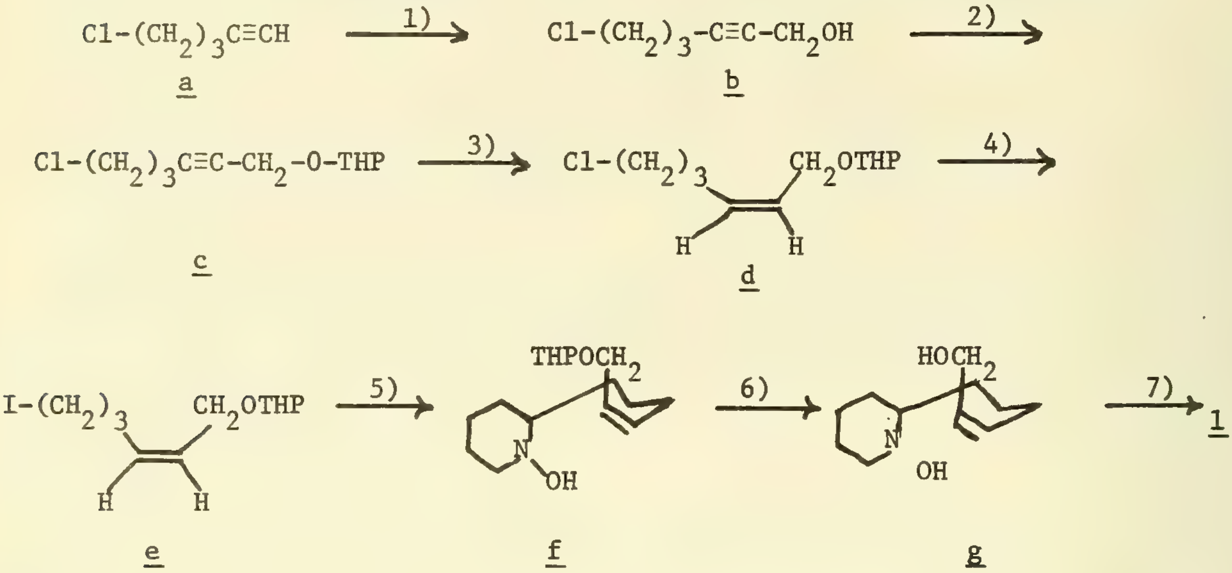
Major Findings: An attractive scheme (proposed by E.G.) for the synthesis of histrionicotoxin analogs substituted in the carbocyclic ring (2) features the intramolecular 1,3-dipolar cycloaddition of the nitrone 1.



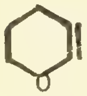
1

2

A synthesis of 1 (R= CH=O) using the following scheme was undertaken.



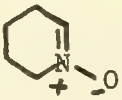
Conditions: 1) EtMgBr, $CH_2=O$

2) 

, TsOH, THP= tetrahydropyranyl

3) $H_2/Pd\text{-}BaSO_4$/Pyridine

4) NaI - Acetone

5) Mg or Li on e + 

6) TsOH, CH_3OH

7) MnO_2 - CH_2Cl_2

Steps 1-- 4 proceeded in excellent yield. Step 5 has failed, in that e fails to react with magnesium metal or gives a dimer with lithium metal. The deblocking (6) and oxidation steps have been conducted in good yield in d. A modified active manganese dioxide has been prepared which prevents cis--trans ismerization in this reaction. This reagent also gives nitrone cleanly from 1-hydroxypiperidine at 0° and should be capable of oxidizing both the hydroxylamine and allylic alcohol functions of g at the same time.

Significance to Biomedical Research and the Program of the Institute: Histrionicotoxin and its analogs have demonstrated utility in probing nerve function. A simple synthesis of related compounds should have great practical consequences.

Proposed Course: Step 5 will be studied further. An exchange reaction of e and CH_3MgI will be attempted. An attempt will be made to block dimer formation in the lithium reaction. Should these and other reactions fail, blocking groups in e different from the THP group (e.g. t-Bu$(CH_3)_2$-Si-) will be tried. Other schemes for building up the olefinic side chain could be attempted.

Keyword Descriptors: Synthesis of Frog Toxin Analogs by Nitrone Cycloadditions.

Honors and Awards: None

Publications: Kuttan, R., Nair, N. G., Radhakrishnan, A. N., Spande, T. F., Yeh, H. J. and Witkop, B.: The Isolation and Characterization of γ-L-Glutamyl-S(*trans*-1-propenyl)-L-cysteine Sulfoxide from Sandal (*Santalum Album* L). An Interesting Occurrence of Sulfoxide Diastereoisomers in Nature. *Biochemistry* 13: 4394, 1974.

Project No. Z01 AM 19404-10 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Modification of Free and Bound Tryptophan

Previous Serial Number: NIAMDD-LC-28

Principal Investigator: Thomas F. Spande

Other Investigators: Bernhard Witkop

Cooperating Units: M. Ohno, Kyushu University, Fukuoka, Japan

Man Years:

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Project No. Z01 AM 19405-02 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Photochemical and Anodic Decarboxylations of Orotidine

Previous Serial No.: NIAMDD-LC-20

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Others:	0

This project has been terminated.

Project No. Z01 AM 19406-02 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Photoinduced Reaction of Uridine with Cyanide Ion

Previous Serial Number: NIAMDD-LC-19

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Others:	0

This project has been terminated.

Project No. Z01 AM 19407-02 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Sustained Action Forms of Biologically Active Nucleosides: 2,2,6-Trimethylcyclohexane-1-carboxylates

Previous Serial Number: NIAMDD-LC-27

Principal Investigator: J. A. Waters

Other Investigator: G. Polansky (S.I.S.)

Cooperating Units: E. De Clercq, Katholicke Universiteit, Te Leuven, Leuven, Belgium

Man Years

Total:	1.0
Professional:	.5
Other:	.5

Project Description:

Objectives: To potentiate the therapeutic level of activity of antitumor, antiviral and immunosuppressant nucleosides.

Methods Employed: Nucleosides have been selectively esterified with 2,2,6-trimethylcyclohexane-1-carboxylic acid using several techniques.

Major Findings: The 5'-monoester of 5'-fluoro-2'-deoxyuridine (FUDR) has been prepared and is now being assayed in tumor cell lines.

Significance to Biomedical Research and the Program of the Institute: These modifications should promote and maintain effective therapeutic levels of the active nucleoside within the cell and thus may be efficacious in the treatment of various cancers, viral diseases and rheumatoid arthritis.

Proposed Course: To extend this study to other candidates such as ara C and 5-IdU.

Keyword Descriptors: Sustained action forms, biologically active nucleosides, antitumor, antiviral, immunosuppressant nucleosides, trimethylcyclohexane-1-carboxylic acid, 5'-fluoro-2'-deoxyuridine, cancer, viral diseases, rheumatoid arthritis.

Honors and Awards: None

Publications: None

Project No. Z01 AM 19408-02 LC
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Analgesics Based on the Piperidine Ring System

Previous Serial Number: NIAMDD-LC-26

Principal Investigator: J. A. Waters

Other Investigator: G. Polansky (S.I.S.)

Cooperating Units: E. L. May, L.C., NIAMDD

Man Years

Total: 1.0
Professional: .5
Other: .5

Project Description:

Objectives: To design and synthesize compounds from the precursor 4-piperidinol and to assay them for analgesic activity and physical dependence liability.

Methods Employed: Numerous synthetic procedures have been used to modify the various positions of the piperidine ring.

Major Findings: 4-Hydroxy-N-methyl piperidine-4-pyrrolecarboxylate exhibits a potency intermediate between that of codeine and morphine in the hot plate assay, shows no morphine-like physical dependence capacity in monkeys and is long acting.

Significance to Biomedical Research and the Program of the Institute: Analgesics, which lack the side-effects of morphine, especially physical dependence capacity, and narcotic antagonists are needed for clinical use in relief of pain and in coping with the drug addiction problem.

Proposed Course: Structural features at various positions of the ring system and analgesic receptor affinities will be examined.

Keyword Descriptors: Analgesics, piperidine ring system, 4-piperidinol, 4-hydroxy-N-methyl piperidine-4-pyrrolecarboxylate, physical dependence capacity, narcotic antagonists, drug addiction problem, analgesic receptor affinities.

Honors and Awards: None

Publications: None

Project No. Z01 AM 19600-10 LC
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Oxidation Mechanisms in Metabolic Processes

Previous Serial Number: NIAMDD-LC-1

Principal Investigator: Louis A. Cohen

Other Investigators: John Reepmeyer and Herman Yeh

Cooperating Units: None

Man Years

Total:	0.5
Professional:	0.5
Other:	0

Project Description:

A study of the mechanisms by which energy derived from respiration is ultimately converted into labile bond energy, as in ATP; a study of the mechanisms by which molecular oxygen is activated for direct incorporation into substrate.

Objectives: To demonstrate that when oxygen is bound to metal-chelate systems, it is transformed from the triplet (diradical) state to the singlet (ionic) state and that the direct incorporation of oxygen into substrate involves the electrophilic attack of activated singlet oxygen. To study possible mechanisms for the conservation of oxidative energy by means of chemical models and to apply such knowledge to elucidation of the enzymatic pathways. To devise chemical models for the Mitchell hypothesis--that ATP formation can be coupled to a pH gradient and the free energy available therefrom.

Methods Employed: Infrared and ultraviolet spectroscopy are used to follow rates of reaction of intermediates and to determine structural characteristics. The latter method is also used for quantitative assay of labile materials. Nuclear magnetic resonance spectroscopy is used for structural elucidation of closely related materials. Various chromatographic procedures, particularly on thin layer plates, are used extensively.

Major Findings: Conformationally constrained imidazolecarboxylic acid esters hydrolyze by way of their cyclic acylimidazoles. Acylimidazoles have been implicated in a variety of other reactions of these systems, providing evidence that high energy bonds can be created enzymatically solely by the trapping of entropic free energy.

Synthesis of the last, but highly elusive, compound in the current series has finally been achieved. As anticipated, this compound shows a high tendency toward utilization of the free energy of water formation as the sole means for generation of high energy bonds. Kinetic studies are in progress to provide a clear model for the Mitchell hypothesis.

Significance to Bio-medical Research and the Program of the Institute: The mechanism by which molecular oxygen is carried into the cell, bound to hemoglobin, has been well elucidated. Its subsequent fate remains obscure. One immediate goal of this project is to determine the mechanism by which oxygen is activated to a state in which it can attack a variety of relatively inert substrates.

Similarly, the function of ubiquinone in oxidative phosphorylation has yet to be elucidated. It has been shown that oxidative energy is utilized in the conversion of ADP to ATP in mitochondria, ubiquinone being an essential cofactor.

The present findings permit a novel role to be assigned to ubiquinone, Vitamin K and other quinone cofactors.

Controversy regarding the Mitchell hypothesis for mitochondrial synthesis of ATP has continued unabated, since no experiment has been devised to test its validity. The model system under study will be used to attempt to settle the question of whether ATP can be synthesized simply by a rapid change in pH.

Proposed Course: Studies will be continued on bond conservation of oxidative processes and efforts will be made to bridge the gap between the chemical model and the mitochondrion. Studies will be continued on the effects of considerable reduction in the electrostatic gap.

Keyword Descriptors: Oxidative phosphorylation, Energy conservation
High energy bonds

Honors and Awards: None

Publications: None

Project No. Z01 AM 19601-08 LC
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemical Modification and Cleavage of Proteins

Previous Serial Number: NIAMDD-LC-2

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: Robert Jerussi, FDA

Man Years

Total: 0.3
Professional: 0.3
Other: 0

Project Description:

A study of the chemical modification and fragmentation of polypeptides and proteins by use of selective chemical and physico-chemical reagents. The elucidation of sequence, tertiary structure and protein topology by such methods. Development of techniques for the selective radiolabeling of membrane proteins.

Objectives: To effect the selective modification and splitting of complex peptides by chemical means. To demonstrate the utility of the techniques in splitting long peptide chains into shorter fragments, facilitating sequence determination. To utilize such methods for selective modification of functional groups on the surface of a globular protein and thus permit the probing of the surface structure. To modify proteins by selective oxidation or reduction techniques.

Methods Employed: Ultraviolet spectroscopy is used to follow the course and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography, ion exchange and high-voltage electrophoresis are used to separate and purify polypeptide fragments. An automatic amino acid analyzer is used to follow the extent of chemical reaction and the purification of peptide fragments. Gas chromatography assists in quantitative end-group assay. Various techniques are used for assay of enzyme activity, including spectral and automatic pH devices. Electrochemical oxidation is effected by use of controlled voltage equipment coupled with automatic pH control.

Major Findings: A technique has been developed for the conversion of carrier-free ^{125}I into the nonvolatile, stable, and powerful iodinating agent, N-iodosuccinimide. Production of this halogen carrier on a large scale will greatly facilitate the technique of radioimmune assay of biological materials.

A soluble polymer containing N-iodosuccinimide units has been developed, which is capable of selective iodination of highly exposed tyrosine and histidine in membrane protein. By use of radioactive iodine in this polymer, the identification of receptor protein may be facilitated.

Significance to Bio-medical Research and the Program of the Institute: Despite the remarkable achievements of the past several years in the X-ray crystallography of proteins, there exists no satisfactory method for determining three-dimensional structure in solution, under conditions of enzymatic activity. The use of a chemically reactive polymer or a platinum electrode, capable of selectively and rapidly modifying functional groups only on the surface, may provide a solution to the problem.

The ability to label membranes in a selective and specific manner with radioactive iodine will serve to facilitate studies on the sites and mechanisms of hormone binding to tissues.

Proposed Course: Information obtained by selective iodination, oxidation and reduction of proteins will be used to provide further information on conformation in solution.

Keyword Descriptors Radioiodination, Iodopolymers, Protein iodination

Honors and Awards: None

Publications: None

Project No. Z01 AM 19602-09 LC
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cleavage of Peptide Bonds by Intramolecular Participation and Mechanisms of Action of Hydrolytic Enzymes

Previous Serial Number: NIAMDD-LC-3

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Project No. Z01 AM 19603-05 LC
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Fluoro Analogs of Enzyme Substrates

Previous Serial Number: NIAMDD-LC-4

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk, John Reepmeyer,
Y. Takeuchi, Herman Yeh

Cooperating Units: Claude Klee, NCI-LC
Jack S. Cohen, NIAMDD-LCP
Dr. Michael Monahan, Salk Institute, LaJolla, California
Dr. Erik De Clercq, Rega Institute for Medical Research,
Leuven, Belgium

Man Years:

Total: 3.4
Professional: 2.9
Other: 0.5

Project Description:

Development of new methods for the synthesis of fluoro analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole had yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: 2-Fluorohistidine has been found a powerful growth inhibitor for E. coli. By mechanisms still unknown, the compound permits multinucleated cells to divide, but prevents duplication of genetic material. 5-Fluoroimidazole-4-carboxamide riboside has been found a highly effective antiviral agent and inhibitor of both DNA and RNA synthesis. 2-Fluorohistamine is an effective agonist for the histamine H1 receptor, but has little or no effect on the H2 receptor. 2-Fluorohistidine analogs of the hormone-releasing factors, TRF and LRF, show 20-30% of the activity of the natural peptides, despite a 5-unit decrease in pK. Methods have been developed for tritium labelling of 4-fluorohistidine. The incorporation of 2-fluorohistidine into animal protein is blocked by actinomycin and cycloheximide, or by administration of a large pool of histidine.

Significance to Bio-medical Research and the Program of the Institute: Past experience with fluoro analogs (fluorocitrate, fluorocortisone, and fluorouracil) warrants a search for other medicinally useful fluoroanalogs. Recent findings on the value of histidine in arthritis therapy, of imidazolecarboxamide in skin cancer therapy, and of histamine as a false neurotransmitter emphasize the magnitude of directions in which fluoroimidazoles may be examined clinically.

Proposed Course: Fluoroimidazoles already prepared will be submitted for pharmacological testing and will be examined as enzyme substrate analogs. Additional and more complex compounds will be prepared for the same purpose. The photochemical method of synthesis will be applied to other types of heterocyclic systems, in addition to imidazole. Fluorine nuclear magnetic resonance spectroscopy will be used to study enzyme-substrate complexes with a view to aiding in the elucidation of mechanism of enzymes such as histidine deaminase.

Keyword Descriptors: Fluoromedicinals, Fluorohistidine, Fluorohistamine
Antivirals, Antibiotics

Honors and Awards: None

Publications:

Dunn, B. M., Di Bello, C., Kirk, K. L., Cohen, L. A., and Chaiken, I. M., Synthesis, Purification, and Properties of a Semisynthetic Ribonuclease S Incorporating 4-Fluoro-L-histidine at Position 12. J. Biol. Chem., 249, 6295-6301, 1974.

Klee, C. B., Kirk, K. L., Cohen, L. A., and McPhie, P.: J. Biol. Chem., in press.

Yeh, H. J. C., Kirk, K. L., Cohen, L. A., and Cohen, J. S.: ^{19}F and 'H Nuclear Magnetic Resonance Studies of Ring-fluorinated Imidazoles and Histidines. J. Chem. Soc., Perkin Trans. 2, in press.

De Clercq, E., Luczak, M., Reepmeyer, J. C., Kirk, K. L., and Cohen, L. A.: Fluoroimidazoles as Antiviral Agents and Inhibitors of Polynucleotide Biosynthesis. Life Sciences, in press.

Project No. Z01 AM 19604-05 LC
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: General Principles of Enzyme Catalysis and Simulation

Previous Serial Number: NIAMDD-LC-5

Principal Investigator: Louis A. Cohen

Other Investigators: Paul S. Hillery, Michael King (part-time Guest Worker)

Cooperating Units: None

Man Years

Total: 2.0
Professional: 1.5
Other: 0.5

Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10}-10^{18}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification.

Major Findings: In order to examine the steric consequences of the trialkyl lock, a series of halogenated lactones have been synthesized, including the severely crowded iodo lock. Studies on the kinetics of opening and closing of these systems are in progress. Halogenated acyclic locked systems have been synthesized, after considerable effort, in order to determine the free energy of the barrier to free rotation in such compounds.

A locked five-membered lactone has been obtained by novel synthesis. The opening and closing of this system has been compared with that of a six-membered lactone; closure to the five-membered lactone is faster, and is the fastest esterification reaction ever observed in aqueous solution at neutral pH.

Significance to Bio-medical Research and the Program of the Institute: Despite the vast amount of research invested in the elucidation of the pathways of specific enzyme-catalyzed reactions, justifications of the rates of these reactions, in comparison with their nonenzymatic counterparts, are still in a primitive state. A sound understanding of the means by which enzymes operate so effectively would lead not only to a better appreciation of biochemical processes in general, but also to a more rational approach to drug design.

Proposed Course: Utilizing the principles of stereopopulation control already developed, additional models will be studied to produce even faster reaction rates than those already observed. The principle will also be extended to other types of reactions, such as hydride transfer and addition/elimination. The control principle will also be used to design conformationally restricted analogs of various drugs with a view to enhancement of activity and reduction of side-effects.

Keyword Descriptors: Enzyme simulation, Enzyme catalysis, Stereopopulation control

Honors and Awards: None

Publications: None

Project No. Z01 AM 19801-30 LC
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Service Function, Section on Microanalytical Services and Instrumentation

Previous Serial No.: NIAMDD, LC, #30

Principal Investigator: David F. Johnson

Other Investigators: P. Parisius, B. Baer, A. Wong, W. R. Landis, H. Y. C. Yeh, N. Whittaker, B. Miller

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description: Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents when requested. Instrumental analyses include: GC/MS spectrometry, gas-liquid chromatography, GC with radioactive monitoring, infrared, nuclear magnetic resonance, atomic absorption spectrophotometry, ultraviolet, and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colormetric, gasometric, coulometric, and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was 13,427, roughly a 20% increase in the total from the last reporting period. Review of the special type of analyses listed below reflects substantial increases in most areas and decreases in only two.

Analyses Run

	1973-74	1974-75	% Change
Carbon	2605	3449	32.4
Hydrogen	2605	3449	32.4
Nitrogen	4833	5105	5.6
Phosphorous	110	136	23.6
Sulfur	67	99	47.8
Halogens	483	263	-45.6
Metals	93	434	366.7
Weight Loss	86	222	158.1
Rotations	13	23	76.9
Miscellaneous	257	247	-3.9
Total	11,152	13,427	20.4

It is apparent that the services are being utilized at a continued high level. The distribution of service rendered is LC (41%); NIH, not including LC (56%); and outside agencies (3%). This distribution reflects virtually no change from last year.

Infrared spectral analysis again has shown a decline for the third year (120-63-30). The 30 samples for this year were specialized techniques such as accurate frequency determination for particular bands, scale expansion for weak bands, dilution work for hydrogen bonding, etc. The decline in this service reflects decreased use by investigators of detailed IR data obtained on our Perkin-Elmer 421. Retention of the service is justified, however, since it is the only instrument available in the LC for occasional detailed studies and only minor attention of personnel is required.

The NMR service includes both the Varian A60 and HA-100. The major share of service work is done on the former. Over 350 spectra were run on the A60 by Mr. Benjamin Miller under the guidance of Mr. Noel Whittaker. Additional spectra were run by individual investigators. Mr. Miller also maintains the documentation and stocking of organic chemical stores for the laboratory. As an additional service function in NMR, Dr. Herman Yeh has run 500 spectra on the Varian HA-100 and 50 on a Varian HR-200 (located in a different section). These specialized spectra were, in addition to Dr. Yeh's research program, reported elsewhere.

Over 1500 samples were submitted for mass spectral analysis on the Hitachi Perkin-Elmer RMU-6E mass spectrometer. This figure represents an increase over requests for the previous year and does not include samples run on the Finnigan system (reported below).

Distribution of samples is as follows:

LC	1203
NIH (Other than LC)	211
Outside NIH (Local Universities)	185
Total	1599

Each analysis on the Hitchi involved several spectral determinations, depending on individual need, including: high resolution mass measurement, involving molecular-ion exact mass determination; isotope quantitative mass-ratio measurement; on-scale spectra recorded either/or the visicorder and the pen recorder; sample mixtures requiring fractional volatility-changing rate separations; and standard qualitative spectra.

In addition to technical mention in manuscripts with NIH and outside investigators, Mr. Landis collaborated with investigators from the Naval Medical Research Institute in a study of Schistosoma Mansoni.

Mr. Noel Whittaker is the principal operator of the Finnigan GC/MS system. This system is a recent addition to the instrumentation group and is used very heavily by the investigators. Approximately 475 samples were run by solid probe in the CI mode. An additional large number of samples were run using GC/MS. These include separation and indentification of amino-acid metabolites from liver homogenates, alkaloid mixtures from frog skin extracts, mixtures of metabolites from bromoanisoles, and mixtures of benzopyrenes, to mention but a few.

As an adjunct service to the Finnigan GC/MS system, we recently acquired a separate Finnigan GC. This allows the investigator to work out chromatographic parameters for separation prior to mass spectral determination. The column can then be transferred into the GC/MS system for analysis. The Finnigan GC has also been interfaced with a Searle RAM (radioactive monitoring) system which allows for radioactive measurement of GC peaks. Use of this RAM system is increasing as its potential becomes known by investigators.

The Section Chief continues to serve as Project Officer on an NIAMDD contract with the Medical Research Council of England. This contract provides reference steroid compounds to qualified research workers in the United States.

Keyword Descriptors: Instrumental analyses, NMR, Mass Spectrometry, Microchemical analysis, GC/MS.

Publications: Austin, F., Frappaolo, P., Gilbert, B., Landis, W., da Rosa M. N., and Stirewalt, M., "Further studies of Schistosoma mansoni cercarial stimulation by crude egg lecithin and other lipids", J. Parasitology 69: 455-463, 1974

Project No. Z01 AM 19802-02 LC
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: 1. Steroid Transformations by *Tetrahymena Pyriformis*
2. β-Hydrosteroid Dehydrogenase from *Tetrahymena Pyriformis*: Preliminary Studies.

Previous Serial Number: NIAMDD, LC, #32

Principal Investigator: Nancy S. Lamontagne

Other Investigators: David F. Johnson

Cooperating Units: C. E. Holmlund, Department of Chemistry, University of Maryland, College Park, Maryland

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description: The protozoan *Tetrahymera pyriformis* does not have an absolute nutritional requirement for steroids, and cannot synthesize them *de novo*. Certain steroids such as progesterone or testosterone have been shown to inhibit the growth rate of *T. pyriformis*; whereas, sterols such as stigmasterol slightly elevate the growth rate. Incubation of *T. pyriformis* with selected sterols results in the introduction of Δ^5, Δ^7, and Δ^{22} double bonds, and the removal of ethyl groups, but not methyl groups, from C-24. Our objective was to determine if steroids that were potentially growth-inhibitory to *T. pyriformis* would be metabolized by this protozoan.

Radio active steroid precursers are added to *T. pyriformis* cultures in the exponential phase of growth. The inoculated cultres are incubated for 24 or 48 hours, and the radioactive products are extracted immediately. Separation and identification of these readioactive products is accomplished by column, thin-layer, and gas-liquid chromatography techniques. If there is a sufficient amount of a product, a mass spectra analysis is also obtained.

All of the substrates incubated (androstenedione, progesterone, 17α-hydroxyprogesterone, testosterone, corticosterone and pregnenolene) have been completely metabolized into multiple products. The most frequently observed

Transformation indicated by thin-layer chromatographic analysis has been the reduction of a 3-ketone to a 3β-hydroxyl. The ability of *T. pyriformis* to effect this Transformation has been confirmed by the identification of 4-androstene-3β,17β-diol as a product from the Testosterone incubations.

The identification of several other radioactive products is being pursued in the hope of establishing that *T. pyriformis* can isomerize a Δ^4-steroid to a Δ^5-steroid, as well as reduce a carbonyl group to a hydroxyl.

Since *Tetrahymena pyriformis* appears to have enzyme systems comparable to those in higher animals, investigation of steroid transformations (and the enzymes involved) in this model system could help in the understanding of steroid metabolism in mammalian systems.

The isolation of 4-androstene-3β,17β-diol as a Transformation product from the incubation of *T. pyriformis* with Testosterone indicates that this protozoan does have a dehydrogenase capable of reducing the 3-ketone of a steroid to a 3β-hydroxyl. Our objective is to demonstrate the presence of a β-hydroxysteroid dehydrogenase (β-HSDH) in *T. pyriformis*, and to purify and characterize the nature of this enzyme.

Initially, the optimal conditions of induction, storage, and stabilizing reagents will be determined for the β-HSDH. These optimal conditions will then be employed in the growth of *T. pyriformis* cultures and extraction of the enzyme preparation from the harvested cells. A variety of standard enzyme purification techniques will be used; the selection of which will be dependent on preliminary determination of the amount and stability of the enzyme. Equilibrium and kinetic studies will be pursued if a sufficient amount of β-HSDH activity can be isolated.

Keyword Descriptors: Microbial Steroid Transformations; *Tetrahymena pyriformis*; Testosterone; β-Hydroxysteroid Dehydrogenase; Enzyme Isolation.

Publications: None

Project No. Z01 AM 19803-02 LC
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: 1) Applications of NMR to the Structural Elucidation of Synthetic Drugs, Metabolites and Natural Products.
2) Nuclear Magnetic Resonance Studies of Reaction Rate, Molecular Motion and Molecular Interaction in Biochemical and Biological Systems.
  a) A Study of the Interaction between Organic Phosphates and Biogenic Amines by ^{31}P NMR.
  b) ^{19}F and ^{1}H Nuclear Magnetic Resonance Studies of Ring-Fluorinated Imidazoles and Histidines.
  c) NMR Study of Benzene Oxide-Oxepin Tautomerism.
  d) An NMR Study of Equilibria and Paramagnetism in a Ferriprotoporphyrin - Cyanide System.

Previous Serial Number: NIAMDD, LC, #31

Principal Investigator: Herman J. C. Yeh

Other Investigators: R. Katz, D. F. Johnson, T. Oh-ishi, A. E. Jacobson, R. S. Wilson, E. L. May, A. M. Jeffery, D. M. Jerina, H. Ziffer, T. F. Spande, K. L. Kirk, L. A. Cohen, H. Yagi, B. Witkop.

Cooperating Units: R. M. DeMarinis, C. H. Foster, D. E. Piccolo and G. A. Berchtold, MIT; R. Kuttan, N. G. Nair and A. N. Radhakrishnan of Christian Medical College Hospital, Tamil Nadu, India; D. T. Gibson, T. R. Patel, J. F. Davey V. Mahadevan, University of Texas at Austin, J. T. Wang, Duquesne University.

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description: Knowledge of molecular structure and stereochemistry of biologically important compounds such as drugs, metabolites and natural products is of great significance to biochemical and biological research. Nuclear magnetic resonance has been used extensively to assign the structures of compounds including the reaction products resulting from reaction of nucleophiles upon arene oxides (known as causative agents in carcinogenesis), metabolites derived from aromatic hydrocarbons, 2,9β-dimethyl-6,7-benzomorphan (having appreciable analgesic activity), and γ-L-glutamyl-S-(trans-1-propenyl) -L-cysteine sulfoxide (isolated from sandal leaves).

The interaction between protonated amines (e.g. biogenic amines, amino acids) and organic phosphate containing subtrates (e.g. Vitamin B_6 phosphates, adenosine triphosphate has been studied by ^1H nmr. Although the results of these studies implied the existence of phosphate-amine interactions, information obtained from ^1H nmr studies, however, offered little evidence about the phosphate-amine direct interaction. Using a $\{^{31}p\}$-^1H nmdr technique and pD titration, we are able to evaluate these interactions quantitatively, thus indicating the greater usefulness of the ^{31}p over the ^1H nmr method for this purpose. Study of the interaction between the phosphate group of 4-deoxypyridoxine-5-phosphate (4-dPNP) and different protonated amines by ^{31}p nmr shows that basic amino acids and biogenic amines have significant interactions with the 4-dPNP. No interactions are observed for acidic or neutral α,β and γ amino acids. Among the 19 biogenic amines studied, norepinephrine shows the strongest interaction with 4-dPNP and mono-methyl phosphate.

Nmr chemical shifts of the ring protons of histidine has been widely used as a probe for the studies of local structure of peptides and proteins. It is known that ^{19}F nucleus is more sensitive than ^1H nucleus for probing the local change effects. ^{19}F and ^1H nmr have been used to investigate the effect of local charge on ^{19}F and ^1H chemicals shifts in 2-fluoro and 4-fluoro histidines, which from some early studies, have indicated their biological significance in analogy to histidine. A large shift in ^{19}F over ^1H nmr signals is observed in both compounds as the side chain groups of these molecules being titrated, illustrative of the high sensitivity of ^{19}F signals to distant field effects. In complex systems, like proteins, the use of ^{19}F as an nmr reporter also offer an advantage over that of ^1H, since overlap or concealment by neighboring signals can be eliminated. Present study suggests that the use of ^{19}F nmr for the study of protein systems is encouraging.

A fast equilibrium between oxide and oxepin tautomers is known to exist in a series of mono- and di- substituted benzene oxides. Variable temperature nmr has been used to study the effects of media and substituents on the positive of equilibrium and to deduce thermodynamic and kinetic parameters of the equilibrium. Preliminary result of this study indicates that the position of the equilibrium depends on solvent and on the position of substitution. Solvent with high dielectric constant or molecules with substitution at the β-position is found to shift the position of equilibrium toward the oxide tautomer.

The subject on the interaction of heme and hemoproteins with ligands has been extensively investigated because of their biological significance. Despite a great deal of investigations about their chemical structure and biological function, little has been determined about the kinetics and mechanism of binding ligands to protoheme. The binding of cyanide ion and imidazole to ferriprotoporphyrin·Cl (hemin) has been under investigation by nmr. The results show that the cyanide ligation to heme is a two step binding process. The first step involves formation of the previously unknown monocyanohemin, and then followed by a dicyanohemin in completion. Binding of imidazole to hemin, however, only results in a dimidazolehemin product. The life time of ligand residing on the hemin molecule is relatively long in comparison to the nmr time scale.

Keyword Description: Applications of NMR to biochemical and biological systems.

Publications: Katz, R., Yeh, H. J. C., and Johnson, D. F.: A quantitative study of organic phosphate-amine interaction by ^{31}p nuclear magnetic resonance. Biochem. Biophys. Res. Comm. 58: 316-321, 1974

Oh-ishi, T., Jacobson, A. E. Wilson, R. S., Yeh, H. J. C., and May, E. L.: Synthesis of 2,9β-dimethyl-5,7-benzomorphan. J. Org. Chem. 39: 1347-1349, 1974.

Jeffery, A. M., Yeh, H. J. C., and Jerina, D. M.: Synthesis of cis-1,2-dihydroxy-1,2-dihydronaphthalene and cis-1,4-dihydroxy-1,4-dihydronaphthalene. J. Org. Chem. 39: 1405-1407, 1974.

Jeffery, A. M., Yeh, H. J.C., Jerina, D. M., DeMarinis, R. M., Foster, C. H., Piccolo, D. E., and Berchtold, G. A.: Stereochemical course in reactions between nucleophiles and arene oxides. J. Amer. Chem. Soc. 96: 6929-6937, 1974.

Kuttan, R., Nair, N. G., Radhakrishnan, A. N., Spande, T. F., Yeh, J. J.C. and Witkop, B.: The isolation and characterization of γ-L-glutamyl-s-(trans-1-propenyl)-1-cysteine sulfoxide from sandal (Santalum Album L). An interesting occurrence of sulfoxide diastereoisomers in nature. Biochem. 13: 4394-4400, 1974.

Jeffery, A. M., Yeh, H. J.C., Jerina, D. M., Patel, T. R., Davey, J. F. and Gibson, D. T.: Initial reactions in the oxidation of naphthalene by pseudomonas putida. Biochem. 14: 575-584, 1975.

Yeh, H. J.C., Kirk, K. L., Cohen, L. A., Cohen, J. S.: ^{19}F and ^{1}H nuclear magnetic resonance studies of ring-fluorinated imidazoles and histidines. J. Chem. Soc. in press, 1975.

Gibson, D. T., Mahadevan, V., Jerina, D. M., Yagi, H. and Yeh, H. J.C.: Oxidation of the carinogens benzo (a) pyrene and benzo (a) anthracene to dihydrodiols by bacteria. Science in press, 1975.

Wang, J. T., Yeh, H. J.C. and Johnson, D. F.: Nmr study of ligand-metalloporphyrin complexation. A study of equilibria and paramagnetism in a ferriprotonporphrin-cyanide system. J. Amer. Chem. Soc. 97: 1968-1970, 1975.

Project No. Z01 AM 19804-02 LC
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Development of Chem. Methods and Synthesis of Chem. Compounds of Use in the Study of Biol. and Med. Problems.

Previous Serial Number: NIAMDD, LC, #36

Principal Investigator: Calvin M. Foltz

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Others: 0

Project Description: The primary goal of this work is to make contributions to the investigation and solution of basic biological and medical problems. The first requirement for the achievement of this goal is comprehensive knowledge of biology and chemistry and of the state of knowledge in various additional areas: such as; the etiology of diseases like atherosclerosis, diabetes, cancer, and mental health; biochemistry; molecular biology; medicinal chemistry; and the chemistry and biochemistry of trace elements. Also essential to the success of this project are intensive reading and study of the current scientific and medical literature, attendance at relevant seminars, and interchanges with other scientists. A substantial expenditure of effort has been and is being made in this area, directed to the establishment and maintenance of an adequate file of notes, references, reprints, etc. to make possible and facilitate future and current research. A wide-ranging knowledge of chemistry, biology, and medical problems coupled with knowledge and understanding of immediate problems which need to be overcome to make further progress in chemistry, biology, and medicine will generate the creative insights and new approaches to effective research experimentation which will contribute to and facilitate investigation of the pressing problems of biology and medicine.

On conducting the research connected with this project the following are applied: standard organic chemical techniques; photochemical procedures; colunm, gas, and thin layer chromatography; UV, IR, NMR, ESR, and mass spectroscopy; and biochemical and isotopic tracer techniques.

The immediate goal under this project is to complete work in progress on the extension of the photocyclization reaction observed with N-chloroacetyl-L-tryptophan {Yonemitsu, Cerutti, and Witkop (1966)} to N-chloroacetyl derivatives of tryptamine, homotryptamine, and 3-(4'aminobutyl) indole. The problems standing in the way of completion of this project appear to have been overcome. The trycyclic lactams produced are of chemical and potential biological and medical interest. The reaction has already been extended to non-heterocyclic bicyclic systems with the photolysis of N-chloroacetyl-2-(α-naphthyl)ethylamine {Foltz, J. Org. Chem., 36, 24 (1971)}. This work is to be continued in the indole and other systems and will yield compounds of chemical, biological, and medical interest and possibly special methods for modifying biological molecules and structures.

As soon as possible, additional work will be done on the mechanism, scope, and applications of the interesting rearrangement observed on photolysis of 3-(3'chloro-2'-oxopropyl)indolin-2-one (Foltz and Kondo, Tetrahedron Letters, (1970), 3163).

In 1957 the discovery of the fact that the dietary agent Factor 3, which prevented dietary liver necrosis in the rate, supplied trace amounts of selenium was reported {Schwartz and Foltz, J. Am. Chem. Soc., 79, 3292 (1957)}. This report has stimulated a large and growing body of work on the biological and nutritional roles of selenium. At that time it was suggested that selenium might act biologically by participating in oxidation-reduction mechanisms. Recently two selenoenzymes and a selenoprotein which do or may participate in such reactions have been reported {reviewed by Stadtman, Science, 183, 915 (1974)}. Developments in this area are being monitored closely, and as opportunities present themselves for expeditious and interesting experimentation, work on the chemistry and biochemistry of selenium is anticipated.

In carrying out work under this project the overriding considerations are to do significant chemical and biochemical research designed to produce compounds, chemical and photochemical methods, and insights of potential application in the investigation and solution of chemical, biological, and medical problems.

Keyword Descriptors: Organic, Heterocyclic, Selenium, and Medicinal Chemistry; Photochemistry; Biochemistry; Chemical Reagents.

Publications: None

Project No. Z01 AM 19805-01 LC
1. Chemistry
2. Microanalytical Services and Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Gonadal Steroid-Stimulated Formation of Cyclic AMP in Incubated Rat Hypothalmus

Previous Serial Number: None

Principal Investigator: B. A. Weissman

Other Investigators: D. F. Johnson, P. Skolnick and J. Daly.

Cooperating Units: None

Man Years:
Total:
Professional:
Other:

Project Description: In the immature female rat, diethylstilbesterol (DES) and estradiol (E_2) elicit significant increases in levels of endogenous and {^{14}C}cyclic AMP in {^{14}C}adenine-labeled hypothaliam. The possible link between the estrogens, catecholamines and cyclic AMP accumulation was studied in vitro. Similar considerations apply to the immature male rat.

As mentioned above, DES and E_2 but not estroil or 17α-estradiol elicited increased cyclic AMP formation. This stimulation could be blocked by α and β adrenergic blockers and the anti-estrogen clomiphene in the female rat. It was also found that higher doses of DES has a dual effect: a) estrogenic and b) possibly a release of adenosine. The accumulation of cyclic AMP could also be blocked in the female rat by slicing the hypothalamus and adding theophylline. In the case of male rats, testosterone and DHT, the major androgens have no significant effect on the adanylate-cyclase system while E_2 has a small effect. On the other hand, DES is very potent in this respect and cannot be blocked by α or β adrenergic blockers as well as dopamine antagonists. Clomiphene too has no effect on the stimulated levels of cyclic AMP elicited by DES.

Based on these results we will now try castrated males and neonately androgenized females. The hypothesis is that castrated males should exhibit more female responses while the androgenized female should lose some of her characteristic responses.

Keyword Descriptions: Gonadal steroids; Hypothalamus; cyclic AMP.

Publications: Weissman, B. A. and Skolnick, P.: Stimulation of adenosine 3',5'-monophosphate formation in incubated rat hypothalamus by estrogenic compounds: Relationship to biologic potency and blockade by anti-estrogens: Neuroendocrinology, in press.

Project No. Z01 AM 20000-08 LC
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Previous Serial Number: NIAMDD-LC-39

Principal Investigator: Cyrus R. Creveling

Other Investigators: J. Daly and A. Rotman

Cooperating Units:
R. Borchardt, University of Kansas, Lawrence, Kansas
B. Highman, Natl. Center Toxicological Res., Jefferson, Arkansas
B. Hartman, Washington University, St. Louis, Mo.
G. Breese, University of North Carolina, Chapel Hill, N. C.
I. Carter, NIAMDD-LBP
L. Tice, NIAMDD-LEP
X. Breakefield, Yale University, New Haven, Conn.
B. Nikodejevic, University Skopje, Yugoslavia
P. Siekevitz, Rockefeller University, N. Y.
R. Cohen, Rockefeller University, N. Y.
J. Lundstrom, Astra Läkemedel AB, Stockholm, Sweden
P. Ghosh, Guest Worker, Howard University, Washington, D. C.

Man Years:

Total: 3.6
Professional: 2.3
Other: 1.3

Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines and their precursors and transformation products. To develop new techniques for the investigation of transport metabolism and binding phenomena as related to these amines and their interactions with various drugs. To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of these enzymes and the effect of pH, temperature, substrate specificity, reversible and irreversible inhibitors and the ionic composition of the incubation media. To develop assays and to apply these enzymatic assays to determine specific biogenic amines and to study the distribution and variation of enzyme levels. To develop new pharmacologically active agents for the study of the biological parameters that affect biogenic amines. To develop immunoassay techniques for the study of enzymes involved in the formation, degradation and activity of biogenic amines.

Methods Employed: Drugs and substrates labeled with tritium, deuterium or ^{14}C have been used to study the metabolic fate of endogenous amines and amino acids and the underlying mechanisms of uptake, release and metabolism of these compounds. Standard methods of enzyme purification have been used including: differential centrifugation, density gradient centrifugation, gel filtration, electrophoresis, standard column chromatography, immunodiffusion, immunoelectrophoresis and affinity labeled column chromatography.

Major Findings: More efficient methods for the isolation of catechol-O-methyl transferase (COMT) utilizing affinity and immunoabsorption chromatography have been developed. The amino acid composition of one form of COMT, mol. wt. 23000, has been determined. The interaction of 6-hydroxydopamine with one of the two sulfhydryl groups present in the active site of COMT has been demonstrated. A radioimmunoassay for COMT has been perfected and used to measure enzyme levels in discrete areas of rab brain. The localization appears to be similar in certain respects to the localization obtained with antibody-peroxidase studies. The peroxidase localization appears to be restricted to membrane-bound forms of COMT and in preparations of rat cortex the loci appear as widely distributed arc like structures which may be similar to the apparent dendritic terminals outlined by immunofluorescent techniques. Electron microscopic studies with ferritin-labeled anti-COMT and highly purified synaptosomes from rat brain tentitatively confirm the localization of membrane-bound COMT at the post-synaptic membrane. Several new approaches to improving the specificity of the ferritin-binding using isolated liver microsomes as a tissue substrate have been developed. Radioactive ferritin was prepared and used to quantitate the binding and negative staining techniques were used to observe the specificity of the binding. Washing the tissue preparation with a lysine buffer markedly reduces the nonspecific binding. Continuing studies on the localization of COMT are in progress.

Studies on the structure/activity correlations on the inhibition of uptake, release and long term effects on the uptake mechanisms by phenethylamine phenethanolamines and indoleamines in heart have been continued. A technique for measuring the uptake of amines by diced suspensions of mouse atria has been developed which includes several technical improvements such as the simultaneous determination of the uptake of tritiated amines and the atrial sample size with the diffusion of carbon-labeled inulin and the essentially instantaneous termination of uptake by the addition of cold fixative to the reaction tube. Control of the amine concentration in this in vitro assay is much more precise than in previous studies which were performed in vivo and permits more accurate estimations of the affinity of adrenergic neurons for amines. Studies of the uptake of tritiated 6-hydroxydopamine and the irreversible finding of the oxidation products of this amine have clearly shown that there is a rapid onset of irreversible binding and a concommitant loss of the uptake of 6-hydroxydopamine and norepinephrine. Similar studies have been carried out with tritiated 5,7-dihydroxytryptamine in an attempt to gain some insight into the previous observation that prior inhibition of MAO blocks the loss of the uptake mechanism and the ensuing neurodegeneration usually seen with 5,7-dihydroxytryptamine.

Routine studies on the inhibition of norepinephrine uptake and the release of norepinephrine are being continued in an attempt to gain further knowledge of the specificity of the uptake mechanism and to find additional compounds which may be useful as investigational tools. Currently we are examining the fluoro derivatives of tyramine and dopamine in this regard. 3-Fluoro tyramine and 6-fluorodopamine are substrates for uptake and displace endogenous norepinephrine.

To examine the effects of 6-hydroxydopamine with regard to mechanism of neurocytotoxicity and to search for model systems of the active uptake mechanism studies were performed in neuroblastoma and other cell cultures. Facilitated uptake of dopamine and 6-hydroxydopamine were obtained in an adrenergic clonal line of neuroblastoma, N1E-115, but no clear evidence was obtained for active uptake. Determination of the extent of total protein binding of 6-hydroxydopamine and the nature of the soluble proteins extracted from these cells revealed that one or more of the oxidation products of 6-hydroxydopamine were covalently bound to protein. There was a marked increase in the proportion of high-molecular weight proteins in these cells and these proteins contained nearly all of the bound 6-hydroxydopamine. This result supports our previous hypothesis derived from model studies with purified proteins in vitro that the mechanism for neurocytotoxicity results from the intraneuronal crosslinking of proteins by one or more of the oxidation products of 6-hydroxydopamine.

A study of the incorporation in protein, distribution and metabolism of ^{3}H-2-fluoro-histidine in mouse is being continued. Incorporation of 2-fluorohistidine into globin in vitro utilizing the rabbit reticulocytic system has been demonstrated. Studies are in progress which indicate that 2-fluorohistidine can substitute for histidine giving rise to globin containing 2-fluorohistine.

Evidence for direct sympathetic innervation of thyroid follicular cells has been obtained by morphological studies of mouse thyroids in conjunction with studies on the uptake of 6-hydroxydopamine, 5,7-dihydroxytryptamine and 5-hydroxydopamine in vivo.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis, uptake and metabolism of biogenic amines are of fundamental importance to the fields of neurochemistry, pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system and the central biogenic amine-containing neurons. The nature of the localization of membrane-bound COMT as a possible component of the post-synaptic membrane has fundamental implications with respect to function of the sympathetic nervous system and may provide insight into the etiology of essential hypertension. An understanding of the mechanism of the uptake and action of cytotoxic agents such as 6-hydroxydopamine will not only provide a rational for the design of additional agents but may possible provide guidelines for the eventual use of such specific agents in man in the treatment of tumors.

Proposed Course of Project: Protein polymerization induced by cytotoxic amines in vitro will be studied to determine to what extent this reaction is the mechanism of cytotoxicity in vivo. Studies on the specificity and nature of the active uptake mechanisms found in the plasma and vesicular membranes of sympathetic neurons with special emphasis on the interaction of radioactive cytoxic amines will be continued. Localization of membrane bound catechol-O-methyltransferase with immunofluorescence immunoperoxidase and immunoferritin techniques to determine both the regional and cellular distribution of this enzyme will be continued. A radioimmunoassay for rodent catechol-O-methyltransferase will be used to determine the regional distribution in the CNS. The induction of increased enzyme levels by nerve-growth factor, pharmacological parameters and receptor supersensitivity induced by electrolytic and chemical denervation will be explored. The binding and release of norepinephrine as influenced by drugs will be explored further, as will interactions of certain amines and drugs at storage and receptor sites. Studies on inhibitors of tyrosine hydroxylase, phenylalanine hydroxylase, dopamine-β-hydroxylase and catechol-O-methyltransferase will be continued. The incorporation of fluorohistidines into protein will be studied with in vitro systems for protein synthesis.

Keyword Descriptors: Biogenic Amines, Uptake Mechanisms, Antibodies, Histochemistry

Honors and Awards: None

Publications:

Rotman, A., Lundstrom, J., McNeal, E., Daly, J. and Creveling, C. R.: Norepinephrine uptake sites in cardiac tissue. Lack of affinity of 6-hydroxynorepinephrine and related compounds. J. Med. Chem. 18: 138-142 1974.

Creveling, C. R., Lundstrom, J., McNeal, E. T., Tice, L. and Daly, J. W.: Dihydroxytryptamines: Effects on noradrenergic function in mouse heart in vivo. Mol. Pharmacol. 11: 211-222, 1975.

Project No. Z01 AM 20001-05 LC
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

Previous Serial Number: NIAMDD-LC-37

Principal Investigator: John Daly

Other Investigators: P. Skolnick, H. D. Mah, K. Dismukes, Y. Ohga, C. R. Creveling, M. Rogers, B.-A. Weissman

Cooperating Units: U. Schwabe, Guest Worker, Medizinische Hochschule, Hannover, W. Germany
F. Bloom, G. Siggins, and B. Hoffer, St. Elizabeths Hospital, NIMH
D. S. Segal, Dept. Psychiatry, University of California at San Diego
J. Schultz, University Tübingen, Tübingen, W. Germany

Man Years:

Total: 4.7
Professional: 3.6
Other: 1.1

Project Description:

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP and cyclic GMP in the central nervous system. To investigate the possible role of cyclic AMP in regulating biochemical reactions and membrane properties in brain tissue.

Methods Employed: Isotopically labeled compounds have been used to study the formation and breakdown of adenine nucleotides in brain slices and with partially purified enzymes.

Major Findings: Methoxamine has been found to be a specific stimulant of α-adrenergic receptors controlling cyclic AMP formation in rat cortical slices. The magnitude of responses of cortical cyclic AMP-generating systems to methoxamine correlated negatively with spontaneous behavioral activity in four strains of rats. Certain of the α-receptors appear activated by norepinephrine only in the presence of adenosine. In rat cortical slices both norepinephrine and methoxamine will elicit repetitive accumulations of cyclic AMP. Thus, the refractoriness to amines after one stimulation which pertains in guinea pig does not pertain in rat (Skolnick).

Clonidine, normally considered a potent α-agonist, has been found to be perhaps the most active known α-antagonist towards norepinephrine-stimulated formation of cyclic AMP in rat cortical slices. In addition, clonidine, oxymetazoline and phenylephrine greatly potentiate the accumulation of cyclic AMP elicited by very low concentrations of the β-agonist, isoproterenol (Skolnick).

In rat cerebellar slices, β-antagonists completely block the accumulation of cyclic AMP elicited by norepinephrine or isoproterenol. Neuroleptics also antagonize the effect of the catecholamines but only by 30-40%. It would appear based on these results and parallel studies on the prevention by β-antagonists and neuroleptics of the inhibitory effects of catecholamines on spontaneous discharge of cerebellar Purkinje cells that norepinephrine-elicited formation of cyclic AMP occurs in at least two cell populations and that only the β-receptors of Purkinje cells are blocked by neuroleptics such as fluphenazine and α-flupenthixol (Skolnick).

Histamine stimulated formation of cyclic AMP in guinea pig cortical slices has been shown to occur primarily through activation of an H_1-receptor, while in hippocampus both H_1- and H_2-receptors are important. Synergism between histamine and adenosine in both brain regions occurs primarily through H_1-receptors. The affinity of histamine analogs for the H_1-receptors is greatly increased by adenosine. Metiamide was used in these studies as a H_2-antagonist, d-brompheniramine and other classical antihistamines as H_1-antagonists, 4-methylhistamine as an H_2-agonist and 2-aminoethylthiazole as an H_1-agonist (Rogers, Dismukes).

Prostaglandins of the E series were shown to stimulate accumulation of cyclic AMP in rat brain slices (neocortex, striatum, midbrain-thalamus-hypothalamus), while prostaglandin $F_{1\alpha}$, A_1, and B_1 were ineffective. Relatively high concentrations ($EC_{50} \simeq 20\mu M$) were required and no antagonists were found. Indeed, morphine potentiated the effect of the prostaglandin. Prostaglandin E_1 appeared to antagonize the accumulation of cyclic AMP elicited by β-adrenergic agonists (Dismukes).

Radioactive 2-chloroadenine was not effectively incorporated into intracellular nucleotide pools of guinea pig cortical slices and no formation of radioactive 2-chlorocyclic AMP could be detected during subsequent stimulations. Thus, of the various adenine and adenosine analogs studied as potential precursors of abnormal cyclic nucleotides in brain slices only 2'-deoxyadenosine led to formation of such a nucleotide, 2-deoxycyclic AMP (Mah).

Glutamate stimulates cyclic AMP formation in guinea pig cortical slices, but relatively high concentrations are required and the response is blocked by the adenosine antagonist, theophylline. Results obtained with other adenosine antagonists, (isobutylmethylxanthine, adenosine deaminase) support the conclusion that glutamate stimulates cyclases in brain slices through adenosine-dependent mechanisms. However, the weak adenosine antagonist, 2-deoxyadenosine, does not block the glutamate-response. Adenine xylofuranoside appears to block the stimulatory effects of all agents tested including biogenic amines. The potentiation of responses to amines by phosphodiesterase inhibitors appears with diazepam, chlordiazepoxide,papaverine and RO 20-1724 probably due in part to inhibition of adenosine reuptake into slices, thus, leading to synergistic adenosine-amine interactions. (Mah)

Basal levels of cyclic AMP and cyclic GMP in guinea pig cortical and cerebellar slices appear dependent on the presence or absence of calcium ions (Ca^{++}) in the incubation medium. Levels of cyclic GMP decrease 2-3 fold in the absence of Ca^{++} in both cortical and cerebellar slices. Readdition of Ca^{++} leads to a rapid increase in cyclic GMP. Further increases can be elicited by a depolarizing agent, veratidine. The increases in cyclic GMP levels in cerebellar slices are not antagonized by theophylline, atropine, diethylglutamate, or strychnine, but are partially blocked by the GABA antagonist, picrotoxin, and to a lesser extent by bicuculline. In cortical slices, absence of Ca^{++} appears to increase adenosine efflux leading to enhanced basal levels and synergistically enhanced responses to norepinephrine. Both basal and norepinephrine-elicited levels of cyclic AMP in Ca^{++}-free medium are reduced by the adenosine antagonist, theophylline. (Ohga)

After neonatal parenteral treatment of rats with 6-hydroxydopamine, noradrenergic innervation of cortex does not develop, while abnormal noradrenergic hyperinnervation of midbrain occurs. The accumulation of cyclic AMP elicited by norepinephrine is enhanced in both brain regions. In cortex this apparently represents a phenomenon similar to that of denervation hypersensitivity, while in midbrain the enhanced response to norepinephrine indicates that the abnormal noradrenergic terminals are indeed functional; i.e., they are associated with norepinephrine-sensitive postsynaptic adenylate cyclases. In midbrain of adult rats in contrast to cortex, destruction of noradrenergic terminals by intraventricular 6-hydroxydopamine or reduction of norepinephrine by reserpine does not lead to "denervation-like" hypersensitivity to norepinephrine. (Dismukes)

In incubated intact rat hypothalami, diethylstilbestrol elicits after a latent period of 40 min a 3-fold accumulation of cyclic AMP. This accumulation is blocked by the estrogen antagonist, clomiphene, and by the catecholamine antagonists, phenoxybenzamine, propranolol and haloperidol. A nonspecific stimulatory effect seen at high concentrations of diethylstilbestrol is apparently mediated by adenosine since it is blocked by theophylline. Low concentrations of estrogenic agents do not elicit accumulations of cyclic AMP in slabs of cerebral cortical tissue or in sliced hypothalami. The results suggest that estrogens evoke after a latent period a release of catecholamines which stimulate postsynaptic cyclases and that this sequence involves intact intrahypothalamic neurones (Skolnick, Weissman).

Preliminary studies demonstrate that lesions of the medium forebrain bundle in rats results in enhanced responses of both norepinephrine and histamine-sensitive cyclic AMP-generating systems in cerebral cortical slices in the lesioned hemisphere. (Dismukes, Ghosh, Creveling)

Significance to Biomedical Research and the Program of the Institute: The key role of cyclases and cyclic AMP and cyclic GMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ.

Proposed Course of Project: The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. Secondary effects due to elevated cyclic AMP levels in brain tissue will be investigated. The in vivo effects of drugs and the effects of lesions and hormonal alterations on the responsiveness of the cyclic AMP system will be further explored. Attempts to label the precursor pool with fluorescent and radioactive adenine analogs will be continued. The nature of the adenosine receptor will be studied further using adenosine analogs modified for possible affinity labeling. Other agents with selective effects on adrenergic, serotonergic and histaminergic systems will be sought. The role of calcium and other ions in the function of cyclic AMP-generating systems will be investigated. Correlations between cyclase responses and behavioral activity will be studied in crosses between rat strains. The effect of viral alteration on cerebellar development on cyclase responses will be probed. Correlations between cyclic GMP and cyclic AMP levels in brain slices will be attempted. Antibodies to proteins involved in the cyclic AMP-generating systems will be prepared (adenylate cyclase, phosphodiesterase-catalytic and regulatory proteins, protein kinase-catalytic and regulatory proteins) and effects of various regimens on the turnover, levels and distribution of these enzymes in brain will be investigated.

Keyword Descriptors: Cyclic Nucleotides, Brain, Neurotransmitters, Receptors

Honors and Awards: None

Publications:

Skolnick, P. and Daly, J. W.: Functional compartments of adenine nucleotides serving as precursors of adenosine 3',5'-monophosphate in mouse cerebral cortex. J. Neurochemistry 24: 451-456, 1974.

Daly, John W.: Cyclic adenosine 3',5'-monophosphate role in the physiology and pharmacology of the central nervous system. Biochem. Pharmacol. 24: 159, 1975.

Dismukes, K. and Daly, J. W.: Norepinephrine-sensitive systems generating adenosine 3',5'-monophosphate: Increased responses in cerebral cortical slices from reserpine-treated rats. Mol. Pharmacol. 10: 933-940, 1974.

Huang, M. and Daly, J. W.: Interrelationships among the levels of ATP, adenosine and cyclic AMP in incubated slices of guinea-pig cerebral cortex: Effects of depolarizing agents, psychotropic drugs and metabolic inhibitors. J. Neurochem. 23: 393-404, 1974.

Weissman, B. and Skolnick, P.: Formation of adenosine 3',5'-monophosphate in incubated rat hypothalamus by estrogenic compounds: Relationship to biologic potency and blockade by anti-estrogens. Neuroendocrinology, in press.

Skolnick, P. and Daly, J. W.: Stimulation of adenosine 3',5'-monosphosphate formation in rat cerebral cortical slices by methoxamine: Interaction with an alpha adrenergic receptor. J. Pharmacol. Exptl. Therap., in press.

Rogers, M., Dismukes, K. and Daly, J. W.: Histamine-elicited accumulations of cyclic adenosine 3',5'-monophosphate in guinea pig brain slices: Effect of H_1- and H_2-antagonists. J. Neurochem., in press.

Dismukes, K. and Daly, J. W.: Altered responsiveness of adenosine 3',5'-monophosphate-generating systems in brain slices from adult rats after neonatal treatment with 6-hydroxydopamine. Exptl. Neurology, in press.

Project No. Z01 AM 20200-07 LC
1. Chemistry
2. Oxidation Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Mechanism of Enzymatic Oxidation and the Role of Reactive Intermediates in Drug Metabolism, Hepatotoxicity and Carcinogenesis

Previous Serial Number: NIAMDD-LC-38

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Man Years:

Total: 6.5
Professional: 6.0
Other: 0.5

Project Description:

Objectives: To study the basic mechanisms of enzymatic oxygen activation and incorporation into organic substrates. To explore enzymatic and non-enzymatic reactions of labile intermediates such as epoxides and peroxides. To devise methods for the synthesis and study of the chemical reactions of arene oxides. To investigate the role of labile oxygenated intermediates in the cytotoxicity and carcinogenicity of xenobiotic substances.

Methods Employed: Isotopically labeled substrates, water, and oxygen gas have been used to study the oxidation of organic compounds to labile metabolites by a variety of enzymes. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystallography and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, high pressure liquid chromatography is being pioneered in this area.

Major Findings: Efforts have continued toward establishing mechanisms and pathways of metabolism of aromatic compounds by enzymatic systems. Pseudomonas putida was demonstrated to stereospecifically form a cis 1,2-dihydrodiol from naphthalene through the action of a dioxygenase based on incorporation of both oxygen atoms from $^{18}O_2$. Both mammalian systems and bacteria convert dihydrodiols to catechols via dehydrogenases. Deuterium labeling studies established that the bacteria dehydrogenase functions by simultaneous removal of both carbinol hydrogens while the mammalian enzyme behaves more like a typical alcohol dehydrogenase. A mutant species of Beijerinckia converts both benzo[a]pyrene (BP) and benzo[a]anthracene to mixtures of cis dihydrodiols. The diols from BP have been identified as cis-7,8-dihydroxy-7,8-dihydro BP by comparison with authentic standards for which chemical routes of synthesis were devised. Two lines of evidence have been pursued which indicate that arene oxides may not be the sole mechanism by which phenols are formed from xenobiotic substances in mammals: i) the first instance primary kinetic isotope effects have been detected, and ii) ratios of phenolic metabolites have been found which are inconsistent with arene oxide intermediates. Metabolism by solubilized and purified hemeproteins (P-450 and P-448) have indicated that cytochrome b_5 and cytochrome b_5 reductase may well play a role in the non-arene oxide pathway proposed. If such a non-arene oxide pathway could be made prominent by induction, the toxic arene oxide pathway could be avoided in mammals. (Jeffrey, Yeh, Yagi, Selander, Tomaszewski, Daly, and Jerina).

Explorations into the fundamental chemistry of arene oxides and polycyclic hydrocarbons have advanced. A multi-step synthesis of 1-hydroxybenzo[a]pyrene has been completed in order that this compound can be tested as a potential carcinogen and mutagen. Reaction of a variety of nucleophiles with benzene oxide has shown that attack occurs not only by simple 1,2-opening, but also by 1,4- and 1,6-attack. The stereochemical course of these reactions is dependent on the nature of the nucleophile. Comparison of the solvolytic chemistry of K-region and non-K-region arene oxides has shown that trans dihydrodiols rather than phenols are formed from K-region arene oxides at physiological pH. This accounts for the failure of previous workers to detect K-region phenols as metabolites from polycyclic hydrocarbons. Careful examination of the structure of mercapturic acid adducts from arene oxides has shown much of the previous literature on this subject to be in error due to unprecedented migrations of the thiol residues on dehydration and aromatization. Photolysis of arene oxides at low temperature (-196°C) has established that keto tautomers of phenols are initial photoproducts. The keto tautomers are of interest since they are precursors to the phenols formed from arene oxides in biological systems. Several years of effort on the synthesis of K-region and non-K-region arene oxides have culminated in the development of highly efficient and convenient methods for the preparation of these compounds. Both types of arene oxides are prepared from halohydrin ester precursors which serve as masked functionality for the oxirane ring (Lindsay-Smith, Jeffrey, Yeh, Yagi, Selander, and Jerina).

The mobilities of 24 potential metabolites of benzo[a]pyrene were examined with high pressure liquid chromatography. Twelve phenols, five quinones, four dihydrodiols, and three oxides were studied. The chromatographic procedure employed allowed the separation and quantitation of benzopyrene metabolites into three major groups consisting of phenols, quinones, and dihydrodiols. Two of the benzopyrene oxides were unstable during chromatography whereas the third oxide was more stable and chromatographed in the quinone fraction. Treatment of rats with phenobarbital or 3-methylcholanthrene enhanced the metabolism of benzopyrene by liver microsomes and altered the relative amounts of the various metabolites formed. In the absence of epoxide hydrase (EC 4.2.1.63), benzopyrene was metabolized primarily to phenols and quinones but was not appreciably metabolized to dihydrodiols by a solubilized reconstituted cytochrome P-448 monooxygenase system. Addition of partially purified epoxide hydrase resulted in the formation of benzopyrene dihydrodiols with a concomitant decrease in the formation of phenolic metabolites, indicating that benzopyrene undergoes metabolism via arene oxides that are precursors for dihydrodiols and phenols. The above study was made possible because of a prior investigation in which epoxide hydrase and cytochrome P-450 were separated by chromatographic procedures (Holder, Dansette, Yagi, and Jerina).

Significance to Biomedical Research and the Program of the Institute: Enzymatic oxidation reactions are extremely important in many biosynthetic pathways and in the metabolism, detoxification and action of most drugs.

Proposed Course of Project: Continued efforts will be directed toward the synthesis of other metabolites of the hydrocarbon benzo[a]pyrene. A wide range of radiochemically labeled arene oxides will be prepared for use as substrates in the study of epoxide hydrase and glutathione-5-epoxide transferase as well as for binding studies to cellular components. New arene oxides of benzo[a]pyrene will be synthesized. Homogeneous epoxide hydrase will be prepared and its mechanism of action examined. Metabolism of polycyclic aromatic hydrocarbons by bacteria will be further elucidated. Both *in vivo* and *in vitro* studies of the biological activity of synthetic benzo[a]pyrene metabolites will be described in the next report.

Keyword Descriptors: Drug Metabolism, Epoxide Hydrase, Cytochrome P-450, Arene Oxides, Mechanisms of Oxidative Metabolism, Benzo[a]pyrene, Carcinogenesis, Bacterial Metabolism.

Honors and Awards:

The efforts of this section continue to be recognized by Hoffmann la Roche in the form of fellowship support for workers in this laboratory. The support has been expanded to provide funds for two fellows during this second year of assistance.

Publications:

Jerina, D. M.: Chemical and biochemical factors which control the toxity and carcinogenicity of arene oxides. In, "Chemical Carcinogenesis" Part A, Ed. P. O. P. Ts'o and J. A. DiPaolo, Marcel Dekker, Inc., New York, 1974 pp. 289-308.

Tomaszewski, J. E., Jerina, D. M. and Daly, J. W.: Cytochrome P-450 monoxygenases and drug metabolism. Annual Reports in Medicinal Chemistry. Vol. 9, Ed. R. V. Heinzelman, Academic Press, New York, pp. 290-299.

Jerina, D. M. and Lindsay Smith, J. R.: Synthesis of benzo[a]pyren-1-01. J. Chem. Soc. Perkin Trans. I: 1702-1706, 1974.

Jerina, D. M., Witkop, B., McIntosh, C. L. and Chapman, O. L.: Photolysis of arene oxides at low temperature. Oxygen walks and the keto tautomers of phenols. J. Am. Chem. Soc. 96: 5578-5580, 1974.

Bjorgo, J., Boyd, D. R., Watson, C. G., Jennings, W. B., and Jerina, D. M.: E-Z-Isomerization in Aldimines. J. Chem. Soc. Perkin II. 1081-1084, 1974.

Holder, G., Yagi, H., Dansette, P., Jerina, D. M., Levin, W., Lu, A. Y. H. and Conney, A. H.: Effects of inducers and epoxide hydrase on the metabolism of benzo[a]pyrene by liver microsomes and a reconstituted system: Analysis by high pressure liquid chromatography. Proc. Nat. Acad. Sci. USA, 71: 4356-4360, 1974.

Jeffrey, A. M., Yeh, H. J. C., Jerina, D. M., De Marinis, R. M., Foster, C. H. Piccolo, D. E. and Berchtold, G. A.: Stereochemical course in reactions between nucleophiles and arene oxides. J. Am. Chem. Soc., 96, 6929-6937, 1974.

Dansette, P. M., Yagi, H., Jerina, D. M., Daly, J. W., Levin, W., Lu, A. Y. H., Kuntzman, R. and Conney, A. H.: Assay and partial purification of epoxide hydrase from rat liver microsomes. Arch. Biochem. Biophys. 164: 511-517, 1974.

Jeffrey, A. M., Yeh, H. J. C., Jerina, D. M., Patel, T. R., Davey, J. F., and Gibson, D. T.: Initial Reactions in the Oxidation of Naphthalene by *Pseudomonas putida*. Biochemistry, 14: 575-584, 1975.

Bruice, P. Y., Bruice, T. C., Selander, H. G., Yagi, H., and Jerina, D. M.: Comparative Mechanisms of Reaction of K-Region and Non-K-Region Arene Oxides of Phenanthrene. J. Am. Chem. Soc. 96: 6814-6815, 1974.

Lu, A. Y. H., Levin, W., Selander, H., and Jerina, D. M.: Liver Microsomal Electron Transport Systems. III. The Involvement of Cytochrome b_5 in the NADPH-Supported Cytochrome P-450-Dependent Hydroxylation of Chlorobenzene. Biochem. Biophys. Res. Commun., 61: 1348-1355, 1974.

Tomaszewski, J. E., Jerina, D. M., and Daly, J. W.: Metabolism of aromatic substrates to phenols by animal mono-oxygneases: Evidence for a direct oxidative pathway not involving arene oxide intermediates.

Jeffrey, A. M., and Jerina, D. M.: Novel Rearrangements During Dehydration of Arene Oxide Adducts. A Reappraisal of Premercapturic Acid Structures. J. Am. Chem. Soc.

Yagi, H. and Jerina, D. M.: A General Synthetic Method for Non-K-Region Arene Oxides. J. Am. Chem. Soc.

Gibson, D. T., Mahadevan, V., Jerina, D. M., Yagi, H., Yeh, H. J. C.: Oxidation of the Carcinogens Benzo[a]pyrene and Benzo[a]anthracene to Dihydrodiols by a Bacterium. Science.

Selander, H. G., Jerina, D. M., and Daly, J. W.: Metabolism of Chlorobenzene with Hepatic Microsomes and Solubilized Cytochrome P-450 Systems. Arch. Biochem. Biophys.

ANNUAL REPORT
LABORATORY OF EXPERIMENTAL PATHOLOGY

Studies in the Section of Molecular Pathology (Dr. George G. Glenner and staff) have continued to be concerned with various aspects of the Pathogenesis of Amyloidosis.

The Nature and Pathogenesis of Amyloidosis: Amyloid deposits of both localized amyloid and systemic amyloidosis have been shown by x-ray diffraction crystallography and infra red spectroscopy to be composed predominantly of fibrils having a β-pleated sheet conformation. The polypeptide chains are aligned antiparallel and perpendicular to the fibril axis in all cases of the systemic disease thus far studied, but in the localized process the polypeptide chains may be aligned in either an antiparallel or parallel fashion. Synthetic fibrils formed from glucagon and insulin have been demonstrated to have all the tinctorial, crystallographic and spectroscopic characteristics of amyloid fibrils; glucagon fibrils being arranged in an antiparallel and insulin fibrils in a parallel β-pleated sheet conformation. These findings have led to the definition of amyloidosis as a disease caused by the physicochemical alteration of a protein precursor leading to the deposition of a β-pleated sheet fibril. (Glenner, Linke, Bladen, Eanes, and Termine).

Amyloid of Immunoglobulin Origin: One large group of cases of systemic amyloidoses usually either associated with myeloma or unassociated with any disease process (i.e. "primary") have been previously shown by amino acid analysis, molecular weight determinations and amino acid sequencing to be composed of fibrils having as their sole protein constituent a homogeneous intact light polypeptide chain and/or its NH_2-terminal variable region fragment. Investigation of the sole protein constituents of the systemic amyloid deposits in a case of IgD myeloma has shown these to be composed of an intact λ-light polypeptide chain and two NH_2-terminal fragments of this light chain, one of 17,500 and one of 12,500 molecular weight. These amyloid fibril proteins were shown to be antigenically similar to a monoclonal λ-light polypeptide chain present in the patient's urine and serum (White, Linke, Glenner). β-pleated sheet fibrils could be created by peptic or tryptic proteolytic cleavage of some intact Bence Jones proteins, but it had not been possible by this method to create such fibrils from Bence Jones proteins associated with patients having amyloidosis. By the use of a renal tubular lysosomal enzyme preparation, however, it has been possible to create by proteolysis β-pleated sheet fibrils from the vast majority of such amyloid-associated light chains. This study has demonstrated that lysosomal proteolysis is one potential pathogenetic mechanism for amyloid fibril formation (Epstein, Osserman and Glenner).

Amyloid of Unknown Origin: A further large group of cases of systemic amyloidosis have a major fibril protein (AA) which is of similar amino acid sequence and has no apparent relationship to any known immunoglobulin. The majority of these patients have amyloidosis associated with another chronic disease process (i.e. "secondary" amyloidosis). There is also present in the serum of these patients, as well as in many patients without amyloidosis, an antigenically related serum protein (SAA). The SAA protein has been found to fractionate in a 200,000±20,000 molecular weight range on G-200 column chromatography in phosphate buffered saline and to have an α_1-α_2 mobility on immunoelectrophoresis. Following denaturation in 5M guanidine-HCl and G-100 column chromatography, antigenic reactivity as determined by immunoprecipitation was found at an elution volume corresponding to cytochrome C and SDS disc gel electrophoresis revealed the SAA protein to have a molecular weight of 12,500 (Linke, Sipe, Ignaczak, Pollock and Glenner). Isolation and purification of this serum protein has been accomplished and amino acid analyses reveal it to be closely related to the tissue amyloid protein (Sipe, Ignaczak, Pollock and Glenner).

A radioimmunoassay using ^{125}I-labelled AA and antibodies made monospecific by chromatography on immunoabsorbents of immobilized AA, purified by isoelectric focusing, has now made possible the identification and quantitation of the SAA protein in sera in amounts corresponding to as low as one nanogram of AA protein. A study of over 700 normal and pathologic sera has revealed elevated levels of this protein in a variety of disease processes related and unrelated to amyloidosis, but the absence of significant levels in normal individuals (Sipe, Ignaczak and Pollock).

The studies in the Section on Chemical Pathology (Dr. Friedman and staff) have been concerned with the control of genetic function in Interferon-treated and Virus-infected animal cells.

Control in Interferon-treated Cells: Treatment of animal cells with the appropriate interferon induces changes in the cell which lead to the establishment of an antiviral state. Most viruses replicate poorly in such cells. The plan is to study what changes interferon brings about and how virus replication is impaired in these cells.

Viral messenger RNA is translated with fidelity in L cell system. In extracts from interferon-treated cells, the translation of viral messenger RNA is inhibited. Current work is directed toward the specificity of this inhibition and the possible role of tRNA in this process. The understanding of the selective translational inhibition induced by interferon treatment may lead to a better understanding of post-transcriptional controls in animal cells.

Inhibition with interferon of RNA tumor virus induction by IDU has also been achieved. This inhibition has been demonstrated in cells infected with a xenotropic agent so that it is likely that the interferon-induced inhibition is directed against virus activation by IDU.

Also, a substantial inhibition of RNA tumor virus production has been observed in a mouse tissue culture line (AKR) which is chronically infected with the virus. This marked inhibition of production of virus was accompanied by an increase in intracellular viral group-specific antigen and no alteration in the level of viral reverse transcriptase activity. Studies of 10 cell lines make it likely that the inhibition of virus production observed is due to an interferon effect on the tissue deposit of a "Congo red birefringent" fibrillar material composed of cells. In addition, prolonged treatment with interferon has resulted in a chronic inhibition of extracellular virus production still accompanied by lack of inhibition of intracellular viral protein synthesis, at least as measured by levels of viral gs antigen and reverse transcriptase activity.

Finally, in a separate study interferon has been shown to inhibit cytopathic effects in virus-infected cell cultures. These cultures were found to multiply eventually to form normal cultures with no evidence of residual virus. In effect these cultures seem to have been "cured" of their virus disease. (Drs. Friedman, Jay, Ramseur, Chang, Myers, and Pinkus)

Control in Virus-infected Cells: Adeno-associated viruses (AAV) are defective parvoviruses containing the smallest known animal virus DNA genome. AAV infection is widespread in humans and recently parvoviruses have been implicated in human hepatitis A and certain forms of acute nonbacterial gastroenteritis. AAV is grown in culture in human cells in association with a helper adenovirus. Molecular studies are being performed to analyse in detail the fine structure and function of the AAV genome. A detailed analysis of the metabolism of AAV specific RNA has been completed. It is concluded that only a single mRNA is synthesized by AAV. This RNA is transcribed from a contiguous region of the AAV DNA minus strand equivalent to 70% of the strand length.

Cleavage of AAV DNA with bacterial restriction endonuclease *EcoRI* yields three fragments A, B and C which have been characterized with respect to molecular weight, genetic complexity, physical order and strand polarity. The fragments A, B and C contain 57%, 38% and 5% respectively of the AAV genome. Fragment B contains the *left* end and A the *right* end of the genome. The direction of RNA transcription from the minus strand is from left to right beginning in fragment B and ending in fragment A. These restriction fragments are being used to map accurately the start and stop points of the RNA transcript.

The major results of these studies are that the polarity of the genome of AAV has been defined with respect to its ends by use of fragment maps obtained by restruction enzyme cleavage. In addition this work provides an accurate transcription map of the genome and defines the direction of transcription. An important concept that has arisen from these studies is that AAV probably contains only a single gene (for its own capsid protein). These studies continue to lay the molecular basis for an understanding of the possible role of parvoviruses in human disease. (Dr. Carter)

Histochemical and Ultrastructural Studies: Methods and Applications

(Dr. Feder and staff, Section on Biophysical Histology)

Beta-glucuronidase as a cell marker: In chimeric (tetraparental) mice made by aggregation of high-glucuronidase (C57BL) and low-glucuronidase (C3H) strains, the mosaicism of various organs was made apparent by means of the histochemical staining method for beta-glucuronidase. The following observations were made. (a) In some tissues the high- and low-glucuronidase cells were clearly distinguished in the chimeric mouse by the large difference in the intensity with which their cytoplasm was stained. In these tissues almost every cell could be assigned to either the high- or low-glucuronidase group. (b) For some cell types there was the unexpected finding that C3H (low-glucuronidase) cells in the chimera contained far more enzyme than the same type of cell in nonchimeric (single-genotype) C3H mice; the phenomenon was observed in both young and old mice. This observation is consistent with the hypothesis that enzyme or proenzyme passes from high-glucuronidase cells to low-glucuronidase cells. (c) In the liver the average mosaic patch size was found to be considerably smaller than had previously been described by Wegmann, who reported that each liver contained about 20 mosaic patches. (d) Finally, the average mosaic patch size was about the same in young and in old mice. (Dr. Feder with Dr. Mullen and Dr. Sidman.)

Marking neurons for light and electron microscopy: Two highly fluorescent tissue-reactive dyes (N-substituted napthalimides) have been developed, tested, and applied experimentally. The dyes were injected in vivo through recording microelectrodes into individual neurons in intact neural tissue (turtle retina; Hermissenda crassicornis neurons in cerebropleural ganglion, statocyst, and eye). The injected neuron was then identified, both in wholemount and section, by its fluorescence. Typically the entire neuron, and in particular its finest processes, were clearly revealed by the dye. Fluorescence was apparent even in sections 1500 Å thick; the marked cell was thus observable with both the light microscope (by fluorescence of the cell) and the electron microscope (by superposition with the light-optical image). Moreover an occasional cell or cluster of cells, not injected themselves but contiguous with the injected cell, were marked with the dye; in many (perhaps all) instances the cell cluster comprises a functional unit. Diffusion of dye from one cell to another may be an indication of electrical coupling between the cells and may take place through channels in gap junctions. The cells of many early embryos are electrically coupled, and perhaps molecules controlling development move by means of the same channels that mediate electrical coupling; the embryo of the medaka fish is currently under study with these dyes. The new dyes have several important applications: anatomical identification by both light and electron microscopy of neurons from which electrical recordings have been made; reconstruction of individual nerve cells in their entirety; and investigation of various related entities - gap junctions, electrical coupling between cells, and diffusion between cells. (Dr. Feder with Mr. Stewart, Dr. Fuortes and Dr. Detwiler.)

Activities in the Section on Cytogenetics (Dr. Tjio and staff) have included several long- and short-term studies in the field of cytogenetics.

Murine spleen transplantation: With collaborators at the University of California in San Francisco (Drs. Brecher and Haley), the characteristics of isologous mouse spleen cells transplanted to the omentum of irradiated and nonirradiated hosts were studied using chromosomal markers. These experiments suggested that splenic stromal cells may facilitate hematopoetic differentiation of stem cells, and further experiments are in progress to study the mechanisms of this phenomenon.

Murine Robertsonian translocations: Balanced translocation carriers are phenotypically normal and fertile, but may have trisomic progeny if carrying two translocations sharing a chromosome arm in common. This observation was utilized to study specific congenital defects associated with trisomy 19 in mice. This subsequently provided a model for other investigators to study the effects of various autosomal trisomy syndromes in mice. Little is known about how chromosome defects affect growth regulation, and our model will be used by several new collaborators to study these problems.

Effects of drugs on human chromosomes: A long-term controlled study of the effects of diazepam (Valium^R) on human peripheral blood chromosomes was completed in collaboration with NIDR. Twenty oral surgery patients were tested before and at four intervals after receiving diazepam. No significant increases in aberration rates were found. There has been controversy about the chromosomal effects of this drug, and the results may be useful in resolving this. (with Dr. Driscoll)

Malnutrition and chromosome damage: Because recent reports claimed that malnutrition in humans and certain laboratory animals produced chromosome damage, we were asked to join a controlled study of the infants of Rhesus monkeys receiving either a normal, protein deficient, or combined protein-carbohydrate deficient diet during gestation. A very low aberration rate in all three groups of Rhesus infants was demonstrated suggesting that under carefully controlled conditions, a relationship between maternal malnutrition and infant chromosomal damage cannot be demonstrated. (with Dr. Sever, NINDS)

Murine tumor viruses: The ability of Abelson virus to initiate oncogenic transformation *in vitro* was studied with chromosomally marked virus-treated cultured spleen cells injected into murine hosts. This study of ascites and solid tumors observed in hosts suggested that the virus may initiate oncogenesis *in vitro*. While C-type RNA viruses are known to induce leukemias and lymphomas, oncogenic transformation of lymphoid cells by such a virus *in vitro* had previously not been reported. (with Drs. Sklar and Rowe, NIAID).

Use of chromosomal banding techniques to study etiology of congenital defects and genetic disorders in humans: The most interesting findings resulting from this ongoing project were the observations of patients with Noonan's syndrome. The phenotype of males and females with this disorder resembles that of Turner's syndrome (45,XO chromosome anomaly). For this reason, there had been disagreement about whether Noonan's resulted from an occult chromosomal anomaly or an autosomal dominant gene with variable penetrance. Since we found no detectable chromosome defect in typical male and female cases using several differential staining techniques (G-, C-, and Q-banding), the latter explanation is favored. Because patients with this relatively common syndrome are frequently fertile, such findings are of importance in genetic counseling.

Recently, several new long-term projects have been initiated:
Primate chromosomes: Chromosome studies of irradiated Rhesus brain tissue cultures are being done. These results, when combined with those of collaborators doing virus isolation, pathological and electron microscope studies

may clarify the mechanism of endothelial cell proliferation observed after radiation-induced brain damage. A series of chromosomal inversions in squirrel monkeys is being studied. Fertility, phenotype and behavioral characteristics of animals from specific matings will be correlated with karyotypic findings. Since inversion heterozygosity in humans may result in offspring with unbalanced karyotypes, these primate studies will provide a model for human defects. (with Dr. Caveness, NINDS and Dr. Symmes NICHD).

Short stature: The frequency of sex chromosome defects in prepubertal and teen-aged girls less than third percentile in height in relation to mid-parental height is being studied. The height standards charts of Tanner *et al.*, U. of London, are used to select cases. So far, 4 of 13 samples analyzed blindly have revealed abnormalities (2 = 45,XO; 1 = 45,XO/46,XX; 1 = 45,XO/46, Xmar+/47,Xmar+mar+), suggesting that short girls with parents of average or above average stature have an increased probability of sex chromosome defects. (Dr. Johnsonbaugh, NNMC).

Chromatid exchange: A new technique for differential staining of chromosomes after *in vitro* replication in the presence of 5-bromodeoxyuridine (BrdU) is being studied. It allows detection and quantitation of exchanges between the sister chromatids of individual chromosomes. Exchange frequency in normal human metaphases under controlled conditions can be established for a given laboratory and then used as a tool to study chromosomal stability in specific diseases, following drug exposure, or to evaluate the effects of other agents.

Observations on structures in thyroid which metabolize catecholamines (Dr. Tice)

Adrenergic nerve endings: In addition to adrenergic nerve endings in the vicinity of blood vessels, structures resembling nerve endings are infrequently observed adjacent to thyroid follicular cells, within the follicular basement membrane. Because these structures take up adrenergic false neurotransmitters (drugs which displace norepinephrine in peripheral adrenergic nerve endings) and undergo degenerative change after administration of neurodegenerative false neurotransmitters, it is believed that they may represent adrenergic nerve endings on thyroid follicles themselves. As such, they afford an anatomical basis for the effects of catecholamines on thyroid function which others have reported.

Parafollicular cells: Because parafollicular cells take up and metabolize certain catecholamines, there is reason to suspect that they might also take up false neurotransmitters, and a series of experiments has been carried out attempting to demonstrate uptake. In the course of these experiments, the effects of acute hypercalcemia on the morphology of the cells was examined, and it was found that extensive degranulation occurred within three hours. However, if mice are chemically sympathectomized with 6-hydroxydopamine, degranulation does not occur after calcium injection, suggesting that the effect of calcium may not be entirely on the parafollicular cells themselves, but may be mediated via some neuronal mechanism. (Dr. Tice and Dr. Creveling, LC-NIAMDD)

Synovial mucin lubrication: Work is being pursued with an ellipsometer at the National Bureau of Standards to try to learn about the adsorbed films of synovial mucin that lubricate the rubbing of joint cartilage surfaces upon each other. (Weeping lubrication reduces the severity of the rubbing but does not eliminate it). The quantity of material in the films is small, so both the chemistry of the adsorption process and the refinements of ellipsometric technique necessary to reduce the uncertainties in the measurements are being pursued. (Dr. Wilkins)

Interference microtopography: The interference microtopography system described in the Journal of the American Optical Society, 37: 1490 (1966) was at that time used with a laser whose beam was swept round and round a narrow cone by a thin wedge prism. The light source had to be on the axis of rotation of the prism, and if more than one wavelength was present their relative deviations were rigidly fixed by refractive indices of the prism. Varying the angle of the cone could only be done by tilting the rotating prism, by making a device that did this.

The rotating small-deviation prism and aligned light have been replaced by a rotating Dove prism and a misaligned light. The Dove prism sweeps the light twice per revolution round a cone whose angle is proportional to the misalignment of the light. Light of different wavelengths can be sent into cones of any desired angle. White light fringes are possible, or repeating rainbow sequences of fringes of high order. It was hoped to use these rainbow fringes to examine the surface roughness of cartilage, but with laser red and mercury-lamp green it is not possible. The mercury lamp is not bright enough. Soon, however, argon-ion lasers will be cheap enough so the method may yet be useful. (Drs. McCutchen and Wilkins)

Fish wake photography: On the strength of stereo-shadograph-of-stratified-fluid method of flow visualization previously developed, Dr. McCutchen has been invited to a restricted conference on the Biodynamics of Animal Locomotion, to be held at Cambridge, England in early September, 1975. An apparatus has been assembled to cine photograph the wakes of small tropical fish. The photography has succeeded, and adequate support for earlier visual observation has been obtained that a swimming fish pushes sideways on the water substantially harder than he pushes backward. The cost in energy is less than one might have expected and is probably paid to avoid even greater losses from muscular irreversibility than would result if he used a hydrodynamically more efficient sort of wiggle. (Dr. McCutchen)

Studies related to the oncogenicity of methylazoxymethanol (MAM) the aglycone of cycasin: Collaborative studies in germfree rats are pursued to explore the role, if any, of the bacterial intestinal flora in the *in vivo* metabolism of 1,2-dimethylhydrazine and azoxymethane following intragastric administration. These two compounds are involved in the synthesis of MAM, the former serving as the starting material, the latter as an intermediate. Both compounds have been shown to be excellent carcinogens for the colon and rectum in animals when parenterally administered to conventional rats. In order to determine the presently still unknown proximate and ultimate carcinogens of each compound a comparative study of the urinary excretory metabolites in conventional and germfree rats has been started. Following collection of urinary specimen and their analysis, the animals are kept for observation and later tumor development. (Dr. Laqueur, Dr. Yamamoto, NCI Mr. McDaniel, LNE, NIAMDD and Dr. Matsumoto, U. of Hawaii)

Project No. Z01 AM 21,000-14 LEP
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies related to the oncogenicity of methylazoxymethanol (MAM), the aglycone of cycasin

Previous Serial Number: NIAMDD-LEP-1

Principal Investigator: Gert L. Laqueur

Other Investigators: Drs. Yamamoto and Matsumoto and Mr. E. G. McDaniel

Cooperating Units: NCI-EXP (Dr. Yamamoto)
LNE-NIAMDD (Mr McDaniel)
University of Hawaii (Dr. Matsumoto)

Man Years:

Total: 4
Professional: 1
Other: 3

Project Description:

Collaborative studies in germfree rats are pursued to explore the role if any of the bacterial intestinal flora in the *in vivo* metabolism of 1,2-dimethylhydrazine and azoxymethane following intragastric administration. These two compounds are involved in the synthesis of MAM, the former serving as the starting material, the latter as an intermediate. Both compounds have been shown to be excellent carcinogens for the colon and rectum in animals when parenterally administered to conventional rats. In order to determine the presently still unknown proximate and ultimate carcinogens of each compound a comparative study of the urinary excretory metabolites in conventional and germfree rats has been started. Following collection of urinary specimen and their analysis, the animals are kept for observation and later tumor development.

Keyword Descriptors:

1,2 dimethylhydrazine
azoxymethane
methylazoxymethanol synthesis
carcinogenesis

Publications:

Laqueur, G. L.: Multiple primary tumors induced with the glycoside cycasin and its aglycone. The 5th Multiple Primary Malignant Tumor Conference on Cancer. Perugia, Italy. April 1973 In press.

Laqueur, G. L. and Spatz, M.: Oncogenicity of Cycasin and Methylazoxymethanol (MAM). In Odashima, S., Sato, H. and Takayama, S. (Eds.): Recent Topics in Chemical Carcinogenesis. Gann Monograph on Cancer Research. Tokyo, Japan. University of Tokyo Press. 1975, Vol. 17. In press.

Project No. Z01 AM 21,001-21 LEP
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Preparation of stained tissue sections for investigation and diagnostic purposes.

Previous Serial Number: NIAMDD-LEP-2

Principal Investigator: Miss Virginia Reveal - Head, Tissue Preparation Laboratory

Other Investigators: None

Man Years:

Total: 2
Professional: 0
Other: 2

Project Discription:

The statistical report of this unit is shown below.

	Specimen Accessioned	Stained Slides		Spare Slides	Total
		Routine	Special		
Animals	451	418			
Surgicals	53	156			
		574	4098		
		4672		4838	9515

Project No. Z01 AM 21,002-10 LEP
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Observations on structures in thyroid which metabolize catecholamines.

Previous Serial Number: NIAMDD-LEP-4

Principal Investigator: Lois W. Tice

Other Investigators: Dr. Seymour Wollman (LP-NCI)
Dr. C. R. Creveling (LC-NIAMDD)

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Adrenergic nerve endings. In addition to adrenergic nerve endings in the vicinity of blood vessels, structures resembling nerve endings are infrequently observed adjacent to thyroid follicular cells, within the follicular basement membrane. Because these structures take up adrenergic false neurotransmitters (drugs which displace norepinephrine in peripheral adrenergic nerve endings) and undergo degenerative change after administration of neurodegenerative false neurotransmitters, it is believed that they may represent adrenergic nerve endings on thyroid follicles themselves. As such, they afford an anatomical basis for the effects of catecholamines on thyroid function which others have reported.

Parafollicular cells. Because parafollicular cells take up and metabolize certain catecholamines, there is reason to suspect that they might also take up false neurotransmitters, and a series of experiments has been carried out attempting to demonstrate uptake. In the course of these experiments, the effects of acute hypercalcemia on the morphology of the cells was examined, and it was found that extensive degranulation occurred within three hours. However, if mice are chemically sympathectomized with 6-hydroxydopamine, degranulation does not occur after calcium injection, suggesting that the effect of calcium may not be entirely on the parafollicular cells themselves, but may be mediated via some neuronal mechanism (with Dr. Creveling).

Keyword Descriptors:

thyroid
parafollicular cells
electron microscopy
catecholamines

Publications:

Tice, L. W.: Effects of hypophysectomy and TSH replacement on the ultrastructural localization of thyroperoxidase. Endocrinology 96: 421-433, 1974.

Creveling, C. R., Lundstrom, E. T., McNeil, E. T., Tice, L. W. and Daly, J. W.: Dihydroxytryptamine: Effects on noradrenergic function in mouse heart *in vivo*. Molec. Pharm. 11: 211-222, 1974.

Tice, L. W.: Ultrastructural localization of peroxidase on pseudopods and other structures of the typical thyroid epithelial cell. Endocrinology 94: 1555-1567, 1974.

Project No. Z01 AM 21,003-07 LEP
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biomechanics and related studies.

Previous Serial Number: NIAMDD-LEP-5

Principal investigator: Charles W. McCutchen

Other Investigators: James F. Wilkins

Man Years:

Total: 1-1/2
Professional: 1-1/2
Other: 0

Project Description:

Synovial Mucin Lubrication. Work is being pursued with an ellipsometer at the National Bureau of Standards to try to learn about the adsorbed films of synovial mucin that lubricate the rubbing of joint cartilage surfaces upon each other. (Weeping lubrication reduces the severity of the rubbing but does not eliminate it). The quantity of material in the films is small, so both the chemistry of the adsorption process and the refinements of ellipsometric technique necessary to reduce the uncertainties in the measurements are being pursued (Dr. Wilkins).

Interference Microtopography. The interference microtopography system described in the Journal of the American Optical Society, 37: 1490 (1966) was at that time used with a laser whose beam was swept round and round a narrow cone by a thin wedge prism. The light source had to be on the axis of rotation of the prism, and if more than one wavelength was present their relative deviations were rigidly fixed by refractive indices of the prism. Varying the angle of the cone could only be done by tilting the rotating prism, a difficult problem solved by Dr. Wilkins.

The rotating small-deviation prism and aligned light have been replaced by a rotating Dove prism and a misaligned light. The Dove prism sweeps the light twice per revolution round a cone whose angle is proportional to the misalignment of the light. Light of different wavelengths can be sent into cones of any desired angle. White light fringes are possible, or repeating rainbow sequences of fringes of high order. It was hoped to use these rainbow fringes to examine the surface roughness of cartilage, but with laser red and mercury-lamp green it is not possible. The mercury lamp is not bright enough. Soon, however, argon-ion lasers will be cheap enough so the method may yet be useful (Dr. McCutchen).

Fish Wake Photography. On the strength, of stero-shadograph-of-stratified-fluid method of flow visualization previously developed, Dr. McCutchen has been invited to a restricted conference on the Biodynamics of Animal Locomotion, to be held at Cambridge, England in early September, 1975. An apparatus has been assembled to cine photograph the wakes of small tropical fish. The photography has succeeded, and adequate support for earlier visual observation has been obtained that a swimming fish pushes sideways on the water substantially harder than he pushes backward. The cost in energy is less than one might have expected and is probably paid to avoid even greater losses from muscular irreversibility that would result if he used a hydrodynamically more efficient sort of wiggle (Dr. McCutchen).

Keyword Descriptors:

Synovial mucin lubrication
ellipsometer
adsorbed films
Interference microtopography
fish wake photography
stero-shadograph-of-stratified fluid method of flow visualization
muscular irreversibility

Publications:

McCutchen, C. W.: Optical exxtent. Applied Optics 13: 1537, 1974.

McCutchen, C. W.: Miniviscometer: A small couette instrument. Biorheology 11: 265-277, 1974.

McCutchen, C. W.: Comment on "Bone articulations as systems of poroelastic bodies". AIAA Journal 12: 256, 1974.

McCutchen, C. W.: Comment on "Some surface characteristics of articular cartilage I and II by V. C. Mow et al. J. of Biomechanics in press.

McCutchen, C. W.: Do mineral crystals stiffen bone by straight-jacketing its collagen. J. Theor. Biology in press.

McCutchen, C. W.: An approximate equation for weeping lubrication solved with an electrical analog. Report of Imperial College Conference on Articular Cartilage. Ann. Rheum. Dis. in press.

Project No. Z01 AM 21,004-02 LEP
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Control of genetic function in interferon-treated and virus-infected animal cells.

Previous Serial Number: NIAMDD-LEP-8

Principal Investigators: Drs. Robert Friedman, Barrie J. Carter, Lawrence Pinkus, Esther Chang, Francis Jay, Maureen Myers, and Janet Ramseur

Cooperating Units: LP, NCI - Drs. A. Rabson, J. Costa and T. Trische
LBV, NIAID - Dr. G. Khoury
McGill University - Dr. D. Denhardt
Yale University - Dr. D. Ward

Man Years:

Total: 11
Professional: 7
Other: 4

Project Description:

Treatment of animal cells with the appropriate interferon induces changes in the cell which lead to the establishment of an antiviral state. Most viruses replicate poorly in such cells. Our plan is to study what changes interferon brings about and how virus replication is impaired in these cells.

Translation of virus specific and cellular messenger RNA's is being studied employing cell-free extracts from interferon-treated and control cells. Polypeptide synthesis is monitored in such systems and partial purification of the factors involved will be attempted.

Studies on the replication of RNA tumor viruses (oncornaviruses) are being carried out in interferon-treated mouse cells. In one system, virus induction from AKR and BALB/C cells by IUDR and other antimetabolites and antibiotics is under investigation; in another, virus replication in RNA tumor virus producing AKR cells is being studied.

Lastly, the fate of interferon-treated cells is currently under study. It had previously been thought that interferon-treated cells which became virus infected were extensively damaged by virus. This resulted in rapid cell death so that virus replication was impaired. The purpose of the current study is to test the hypothesis by infecting interferon-treated cells with high multiplicities of virus and following their fate. Chronic treatment with interferon is also being studied.

Inhibition in cell-free systems. Inhibition of virus-directed translation in extracts from interferon-treated cells has been reported from several laboratories. We are currently investigating the possible role of tRNA in this inhibition. Results suggest that the reported inhibition is reversed by purified tRNA preparations (Pinkus, Jay, Friedman).

Inhibition of RNA tumor viruses. Interferon inhibits production of RNA tumor virus in chronically infected mouse cell cultures. This inhibition differs in two major respects from that usually observed in interferon-treated cell cultures: 1) cultures already producing virus are inhibited; and, 2) there appears to be no inhibition of the production of two virus structural proteins which have been studied - the viral p30 (gs) antigen and the viral reverse transcriptase. In the case of the p30 antigen the level is actually increased in interferon-treated cells.

In addition current studies have shown that of 10 chronic RNA tumor virus-producing culture systems studied, 7 are sensitive to interferon and 3, relatively insensitive. In these systems, interferon treatment inhibits RNA tumor virus production in the 7 sensitive systems, but not in the 3 which are insensitive. Since one of the insensitive systems is a clone derived from a sensitive cell line, this probably means that the inhibition of RNA tumor viruses is a property of the cells and not of the viruses.

In addition, current preliminary results suggest that prolonged treatment of the cells with interferon results in prolonged inhibition of virus replication. This inhibition can be reversed by washing off the interferon.

Finally, interferon-induced inhibition of RNA tumor virus induction by IDU has been demonstrated. We have been able to show that this inhibition extends to xenotropic viruses so that we are not simply blocking a reinfection of the system by viruses, the induction of which interferon may not affect (Ramseur, Mottram, R. Carter, Jay, Chang, Myers, Friedman).

Fate of interferon-treated cells. Interferon-treated cells infected with high multiplicities of a very cytopathic RNA virus (VSV) have been found to recover and grow to form normal cultures. If interferon treatment is continued the titers of virus gradually decline to undetectable levels. So far various methods employed to detect occult virus infection have failed to yield virus. The cultures would appear to have been "cured" by interferon treatment. This may have important implications in so far as it suggests the extent to which cells may be protected by interferon treatment (Friedman, Costa).

Adeno-associated viruses. Adeno-associated viruses (AAV) are defective parvoviruses. Because of the small size of their DNA genomes and unique mode of segregation of complementary DNA strands to separate virions, the AAV offer a unique situation amenable to biochemical analysis. In addition, AAV infection is widespread in humans, and they are therefore important as potential human pathogens. The objectives of this study are:

To analyse the structure of the AAV genome and obtain a transcription map.
To study structure and metabolism of AAV messenger RNA.

AAV is grown in human cells which are coinfected with a second helper virus (adenovirus). Viral DNA and RNA are labeled by incorporation of radioactive precursors and purified by standard procedures. The conformation of RNA and DNA is analysed by ultracentrifugation, gel electrophoresis, molecular hybridization, affinity chromatography, and specific cleavage with restriction endonucleases.

Adenovirus-associated virus (AAV)-specific RNA present in the cytoplasm of cells coinfected with helper adenovirus was analyzed by sucrose gradient sedimentation and gel electrophoresis. In aqueous conditions both gels or gradients revealed three AAV RNA components corresponding to 30S, 27S, and 20S and having apparent molecular weights of 2.6 x 10^6, 1.75 x 10^6 to 1.8 x 10^6, and 0.9 x 10^6 to 1.0 x 10^6, respectively. In nonaqueous, denaturing solvents only the 20S AAV RNA species was observed. For this reason, and because they would be apparently significantly larger than a single AAV DNA strand, both the 30S and 27S species are believed to result from conformational or aggregation effects in the aqueous nondenaturing systems. It is concluded that only a single RNA molecule having a molecular weight of approximately 0.9 x 10^6 to 1.0 x 10^6 is synthesized by AAV.

The single AAV mRNA is transcribed from a contiguous region of the minus strand DNA equivalent to 70% of the strand length. Current studies employing cleavage of AAV DNA with bacterial restriction endonucleases will allow more precise mapping of the stably transcribed region of the AAV genome.

The cleavage of adenovirus-associated virus type 2 (AAV2) DNA by the restriction endonuclease R·EcoRI was investigated using neutral and alkaline sucrose sedimentation electrophoresis in composite agarose-acrylamide gels and reassociation kinetics. Linear monomeric AAV2 DNA duplexes were cleaved at two specific sites to yield three fragments A, B and C each of which is equivalent to a unique region of the genome of approximately 57.2, 38.2 and 4.6% respectively. Cleavage of oligomeric forms of AAV2 DNA (including linear and circular oligomers and network-like structures) yielded fragments A, B and C and two additional components, AB' and AB. At least part of both AB' and AB appear to consist of fragments A and B joined by cohesive ends. The remaining portion of both AB' and AB apparently consists of more bizzare structures such as branched molecules (originating from DNA networks) or they may contain single-stranded regions. Radioactive labeling of the 5' termini of AAV DNA prior to cleavage with R·EcoRI showed that A and B are terminal fragments and C is internal. Separation of the complementary strands of fragments A and B showed that A contains the 5' terminus of the minus strand and the 3' terminus of the plus strand and conversely for fragment B. The physical map of the AAV R·EcoRI fragments can thus be unambiguously determined and is drawn with B at the left and A at the right-hand end. On this map, transcription of stable AAV mRNA from the minus strand proceeds from left to right, beginning in fragment B and terminating in fragment A.

Keyword Descriptors:

interferon, RNA tumor viruses, cell-free protein synthesis, tissue culture, radioimmunoassay, adenovirus-associated virus, transcription map, restriction endonucleases, messenger RNA

Publications:

Carter, B. J.: Analysis of parvovirus mRNA by sedimentation and electrophoresis in aqueous and non-aqueous solution. J.Virol. 14: 834-839, 1974.

Carter, B.J. and Rose, J.A.: Transcription in vivo of a defective parvovirus: sedimentation and electrophoretic analysis of RNA synthesized by adenovirus-associated virus and by its helper adenovirus. Virology 61: 182-199, 1974.

Carter, B.J. and Khoury, G.: Specific cleavage of adenovirus-associated virus DNA by restriction endonuclease R·EcoRI characterization of cleavage products. Virology 63: 523-538, 1975.

Friedman, R.M.: Interferon research in the Red Queen's kingdom. Arch.Pathol. 98: 73-75, 1974.

Friedman, R.M. and Ramseur, J.M.: Inhibition of murine leukemia virus production in chronically infected AKR cells: A novel effect of interferon. Proc.Nat.Acad.Sci.USA 71: 3542-3544, 1974.

Project No. Z01 AM 21,005-08 LEP
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Histochemistry: principles, methods, and applications

Previous Serial Number: NIAMDD-LEP-9

Principal Investigator: Ned Feder

Other Investigators: Peter B. Detwiler, M. G. F. Fuortes, Richard Mullen, Richard L. Sidman and Walter W. Stewart

Cooperating Units: Harvard Medical School (Mullen, Sidman)
LPN-NINDS (Detwiler, Fuortes)

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Beta-glucuronidase as a cell marker. In chimeric (tetraparental) mice made by aggregation of high-glucuronidase (C57BL) and low-glucuronidase (C3H) strains, the mosaicism of various organs was made apparent by means of the histochemical staining method for beta-glucuronidase. The following observations were made. (a) In some tissues the high- and low-glucuronidase cells were clearly distinguished in the chimeric mouse by the large difference in the intensity with which their cytoplasm was stained. In these tissues almost every cell could be assigned to either the high- or low-glucuronidase group. (b) For some cell types there was the unexpected finding that C3H (low-glucuronidase) cells in the chimera contained far more enzyme than the same type of cell in non-chimeric (single-genotype) C3H mice; the phenomenon was observed in both young and old mice. This observation is consistent with the hypothesis that enzyme or proenzyme passes from high-glucuronidase cells to low-glucuronidase cells. (c) In the liver the average mosaic patch size was found to be considerably smaller than had previously been described by Wegmann, who reported that each liver contained about 20 mosaic patches. (d) Finally, the average mosaic patch size was about the same in young and in old mice. (With Dr. Mullen and Dr. Sidman.)

Marking neurons for light and electron microscopy. Two highly fluorescent tissue-reactive dyes (N-substituted naphthalimides) have been developed, tested, and applied experimentally. The dyes were injected *in vivo* through recording microelectrodes into individual neurons in intact neural tissue (turtle retina; *Hermissenda crassicornis* neurons in cerebropleural ganglion, statocyst, and eye). The injected neuron was then identified, both in wholemount and section, by its fluorescence. Typically the entire neuron, and in particular its finest processes, were clearly revealed by the dye. Fluorescence was apparent even in sections 1500 A thick; the marked cell was thus observable with both the light microscope (by the fluorescence of the cell) and the electron microscope (by superposition with the light-optical image). Moreover an occasional cell or cluster of cells, not injected themselves but contiguous with the injected cell, were marked with the dye; in many (perhaps all) instances the cell cluster comprises a functional unit. Diffusion of dye from one cell to another may be an indication of electrical coupling between the cells and may take place through channels in gap junctions. The cells of many early embryos are electrically coupled, and perhaps molecules controlling development move by means of the same channels that mediate electrical coupling; the embryo of the medaka fish is currently under study with these dyes. The new dyes have several important applications: anatomical identification by both light and electron microscopy of neurons from which electrical recordings have been made; reconstruction of individual nerve cells in their entirety; and investigation of various related entities -- gap junctions, electrical coupling between cells, and diffusion between cells. (With Mr. Stewart and Drs. Fuortes and Detwiler.)

Cilia in the statocyst. In the statocyst (a mechanoreceptive organ) of *Hermissenda crassicornis*, the hair cells were shown to have several hundred cilia with typical ultrastructure, including ciliary fibrils with the characteristic 9+2 arrangement. (With Mr. Stewart and Drs. Fuortes and Detwiler.)

Keyword Descriptors:

neurons
light microscopy
fluorescent dyes
naphthalimides

ciliary structure
electron microscopy

beta-glucuronidase
mosaicism
chimeric mice

Project No. Z01 AM 21,006-19 LEP
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein and proteolytic enzyme interrelationships in normal and disease states.

Previous Serial Number: NIAMDD-LEP-11

Principal Investigator: George G. Glenner, M.D.

Other Investigator: P. Scott Pollock, M.D., Clinical Associate (July 1, 1974-June 30, 1975)

Cooperating Units: H. Bladen, Ph.D. (NIDR-LHP)
D. Eanes, Ph.D. (NIDR-LHP)
J. Termine, Ph.D. (NIDR-LHP)
G. C. White, M.D., University of North Carolina, School of Medicine, Chapel Hill, North Carolina
W. V. Epstein, M.D., University of California, School of Medicine, San Francisco, California

Man Years:

Total: 5
Professional: 2
Other: 3

Project Description:

Synthetic formation of β-pleated sheet fibrils. Amyloid deposits of both localized amyloid and systemic amyloidosis have been shown by x-ray diffraction crystallography and infrared spectroscopy to be composed predominantly of fibrils having a β-pleated sheet conformation. The polypeptide chains are aligned antiparallel and perpendicular to the fibril axis in all cases of the systemic disease thus far studied, but in the localized process the polypeptide chains may be aligned in either an antiparallel or parallel fashion. Synthetic fibrils formed from glucagon and insulin have been demonstrated to have all the tinctorial, crystallographic and spectroscopic characteristics of amyloid fibrils; glucagon fibrils being arranged in an antiparallel and insulin fibrils in a parallel β-pleated sheet conformation. These findings have led to the definition of amyloidosis as a disease caused by the physicochemical alteration of a protein precursor leading to the deposition of a β-pleated sheet fibril. β-pleated sheet fibrils could be created by peptic or tryptic proteolytic cleavage of some intact Bence Jones proteins, but it had not been possible by this method to create such fibrils from Bence Jones proteins associated with patients having amyloidosis. By the use of a renal tubular lysosomal enzyme preparation, however, it has been possible to create by proteolysis β-pleated sheet fibrils from the vast majority of such amyloid-associated light chains. This study has demonstrated conclusively that lysosomal proteolysis is one potential pathogenetic mechanism for amyloid fibril formation.

Amyloidosis of immunoglobulin origin. One large group of cases of systemic amyloidosis usually either associated with myeloma or unassociated with any disease process (i.e. "primary") has been previously shown by amino acid analysis, molecular weight determinations and amino acid sequencing to be composed of fibrils having as their sole protein constituent a homogeneous intact light polypeptide chain and/or its NH_2-terminal variable region fragment. Investigation of the sole protein constituents of the systemic amyloid deposits in a case of IgD myeloma has shown these to be composed of an intact λ-light polypeptide chaim and two NH_2-terminal fragments of this light chain, one of 17,500 and one of 12,500 molecular weight. These amyloid fibril proteins were shown to be antigenically similar to a monoclonal λ-light polypeptide chain present in the patient's urine and serum and are presumed to derive from it by lysosomal proteolysis and coprecipitation to form a β-pleated sheet fibril. These findings have led to a rational approach to the chemotherapy of this lethal disease (Ann. Int. Med. 82: 466, 1975).

Amyloidosis of unknown origin. A further large group of cases of systemic amyloidosis have a major fibril protein (AA) which is of similar amino acid sequence and has no apparent relationship to any known immunoglobulin. The majority of these patients have amyloidosis associated with another chronic disease process (i.e. "secondary" amyloidosis). There is also present in the serum of these patients, as well as in many patients without amyloidosis, an antigenically related serum protein (SAA). The SAA protein has been found to fractionate in a 200,000 ± 20,000 molecular weight range on G-200 column chromatography in phosphate buffered saline and to have an α_1-α_2 mobility on immunoelectrophoresis. Following denaturation in 5 M guanidine-HCl and G-100 column chromatography, antigenic reactivity as determined by immunoprecipitation was found at an elution volume corresponding to cytochrome C, and SDS disc gel electrophoresis revealed the SAA protein to have a molecular weight of 12,500. Isolation and purification of this serum protein has been accomplished following monitoring by radioimmunoassay and amino acid analyses reveal it to be closely related to the tissue amyloid protein.

Keyword Descriptors:

amyloidosis, protein conformation, protein purification, lyscsomal proteolysis

Honors and Awards: Guest speaker: Symposium on Amyloidosis, Helsinki, Finland August, 1974.

Publications:

Glenner, G.G.: Nature et Pathogénie de l'Amylose Généralisée. In: Actualitiés Néphrologiques de l'Hôpital Necker. Paris, Flammarion Médecine - Sciences, 1974, pp. 283-290.

Glenner, G.G.: Amyloidosis of Immunoglobulin Origin: Its Nature and Pathogenesis. In Raspé, A. (Ed.): Advances in the Biosciences. Vieweg, Pergamon Press, 1974, pp. 313-321.

Glenner, G.G.: The Nature and Pathogenesis of Systemic Amyloidosis. In: Advances in Nephrology. Chicago, Year Book Medical Publishers, 1974, pp. 291-300.

Glenner, G.G., Terry, W.D. and Isersky, C.: Amiloidosi: Natura e patogenesi. Aggiornamenti Ematologia II: 180-212, 1974.

Glenner, G.G., Eanes, E.D., Bladen, H.A., Linke, R.P. and Termine, J.D.: β-pleated sheet fibrils: A comparison of native amyloid with synthetic protein fibrils. J.Histochem.Cytochem. 22: 1141-1158, 1974.

Kwiatkowska, J., Torain, B. and Glenner, G.G.: A pyrrolidonecarboxylate peptidase from the particulate fraction of Klebsiella cloacae. Purification of the stable enzyme and its use in releasing the NH_2 terminus from pyrrolidonecarboxylyl peptides and proteins. J.Biol.Chem. 249: 7729-7736, 1974.

Linke, R.P., Sipe, J.D., Pollock, P.S., Ignaczak, T.F. and Glenner, G.G.: Isolation of a low molecular weight serum component antigenically related to an amyloid fibril protein of unknown origin. Proc.Nat.Acad.Sci.USA 72: 1473-1476, 1975.

Glenner, G.G., Linke, R.P. and Pollock, P.S.: Systemic Amyloidosis and Immunoglobulins. In Wegelius, O. and Pasternack, A. (Eds.): Symposium on Amyloidosis. New York, Academic Press (in press).

Linke, R.P., Eanes, E.D., Termine, J.D., Bladen, H.A. and Glenner, G.G.: Formation of Amyloid-like Fibrils From Insulin and Glucagon In Vitro. In Wegelius, O. and Pasternack, A. (Eds.): Symposium on Amyloidosis. New York, Academic Press (in press).

Pollock, P.S. and Glenner, G.G.: An alternative method for removing low molecular weight proteins from urea and guanidine HCl solutions. Anal.Biochem. (in press).

Glenner, G.G.: Current concepts of the formation and composition of amyloid. Ann.Clin.Lab.Sci. (in press).

White, G.C., Jacobson, R.J., Binder, R.A., Linke, R.P. and Glenner, G.G.: Immunoglobulin D myeloma and amyloidosis. Immunochemical and structural studies of Bence Jones and amyloid fibrillar proteins. Blood (in press).

Project No. Z01 AM 21,007-04 LEP
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunochemical and biochemical analyses of proteinaceous substances in normal and disease states.

Previous Serial Number: NIAMDD-LEP-14

Principal Investigator: Jean D. Sipe, Ph.D.

Other Investigators: Thomas F. Ignaczak, M.D., Clinical Associate (July 1, 1974-June 30, 1975)

Cooperating Units: Pedro P. Costa, M.D., Instituto de Neurologia, Hospital de Sto. Antonio, Porto, Portugal
Myron Waxdal, Ph.D. (NIAID-LI)

Man Years:

Total: 2
Professional: 2
Other: 0

Project Description:

Human Amyloid of Unknown Origin (AA):

Detection of antigenically related serum component by solid phase radioimmunoassay. There is present in the serum of many patients with chronic disease, both with and without amyloidosis, a component (SAA) antigenically related to the major fibrillar protein (AA) from secondary amyloid deposits. The isolation of SAA has been greatly hampered by the lack of a quantitative assay; attempts at purification have of necessity relied on immunoprecipitation for identification of antigenically active fractions. A solid phase radioimmunoassay (RIA) has been developed to detect SAA in both whole and fractionated serum, based on its cross reactivity with ^{125}I-AA. Chromatographic isolation and characterization of SAA has been greatly facilitated by use of this assay. Analysis of more than 500 serum samples from normal individuals and patients has yielded a distribution of cross-reactivity as follows:

Disease	Median AA Cross-Reactivity, ng/ml
Secondary Amyloidosis	470
FMF	220
Leukemias	63

Disease	Median AA Cross-Reactivity, ng/ml
Myelomas	45
Solid Tumors	44
Non-Hodgkin's Lymphomas	33
Collagen Vascular	26
Hodgkin's Disease	19
Cardiovascular	17
Primary Amyloidosis	17
Neurologic	17
Non-neoplastic Hematologic	16
Metabolic	16
Psychiatric	13
Normal Donors	8

As expected, the highest degree of cross-reactivity was observed with sera from secondary amyloid and FMF patients and the lowest from psychiatric patients and normal donors.

Anti-AA antibodies. The successful immunization of rabbits with AA protein (Linke, Sipe, Pollock, Ignaczak and Glenner, 1975) has been repeated and again peak antibody production has been observed at weeks 15 and 16 of the immunization regimen. It has been found that the amount of initial innoculum (3 mg is better than 1.5 mg) and that the sites of initial innoculation are critical. Application of the RIA to the problem of isolation of SAA by affinity chromatography has shown that SAA is tenaciously bound by immobilized antibodies and requires strong denaturants for elution. Control experiments have shown that non-antigenic proteins strongly bind non-specifically and are also eluted under these conditions. Affinity chromatography, therefore, does not appear to be a promising tool for SAA purification. However, a method for the chromatographic isolation of SAA, utilizing the RIA method with anti-AA antibodies to monitor its fractionation, has been developed in our laboratory. The chemical and physical characterization of SAA is in progress in order to evaluate the pathologic events leading to its deposition in tissues as a β-pleated sheet fibril.

Cross-Reacting Serum Antigen to Murine Amyloid Fibril (SMA) Protein:

A protein of α mobility was previously detected by immunoelectrophoresis with purified anti-SMA antibodies in the serum of young and amyloidotic GP mice, but not in the serum of C3H mice. GP mice develop amyloidosis "spontaneously" (SMA), but amyloidosis must be induced in C3H mice (IMA). A protein antigenically related to SMA and of only slightly higher molecular weight, has been observed in the serum of an amyloidotic CP mouse. The method employed for this demonstration was SDS polyacrylamide gel electrophoresis of double antibody immunoprecipitates between goat anti-rabbit IgG, purified anti-SMA IgG or control rabbit IgG and GP serum or denatured SMA. Thus there may be a putative fibril precursor in murine serum similar to the cross-reacting antigen, SAA, observed in human serum. In this case the murine model may serve as an experimental tool for evaluating the pathogenesis and subsequent treatment of the human disease process.

Immobilized Pyrrolidonecarboxylate Peptidase (PCA'ase):

Previously, immobilized PCA'ase was shown to be highly active toward dipeptides. However, it has now been observed to be inactive toward lambda light chains of molecular weight 25,000 and this is believed to be due to diffusional restrictions imposed upon larger substrates by the immobilizing matrix. Since solid phase PCA'ase would be of value in protein structure studies because enzyme background would be eliminated from sequencing and the enzyme would be reuseable; it is planned to use a radiolabeled polypeptide as substrate to improve sensitivity for kinetic studies and to immobilize the enzyme to a reversibly soluble carrier to minimize diffusional problems.

Keyword Descriptors:

amyloidosis, radioimmunoassay, affinity chromatography, protein structure

Publications:

Linke, R.P., Sipe, J.D., Pollock, P.S., Ignaczak, T.F. and Glenner, G.G.: Isolation of a low molecular weight serum component antigenically related to an amyloid fibril protein of unknown origin. Proc.Nat.Acad.Sci.USA (in press).

Project No. Z01 AM 21,008-09 LEP
1. Experimental Pathology
2. Cytogenetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cytogenetics

Previous Serial Number: NIAMDD-LEP-12

Principal Investigator: Dr. J.-H. Tjio

Other Investigators: Dr. B. J. White

Cooperating Units: NCI-LPHY (Dr. W. W. Smith)
University of California Medical Center, San Francisco (Dr. G. Brecher)
University of California, San Diego (Dr. G. Sato)
University of Colorado Medical Center, Denver (Dr. T. T. Puck)
Harvard Medical Unit, Boston City Hospital (Dr. V. V. Likhite)

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Chromosome identification and studies of chromosomal abnormalities in the mouse and human using differential staining techniques. The differential staining techniques of Giemsa-, quinacrine mustard-, and centromeric heterochromatin-banding are employed to study normal chromosomes and to detect variations in both the human and laboratory mouse karyotypes. The technique of reverse-banding, originally developed in Europe, is currently being studied in our laboratory. This method of chromosomal banding will provide us with another and a different way of determining if a chromosomal abnormality is present. The use of one or more of these techniques provides more information to help us in determining whether or not a karyotypic variation is significant.

Chromosome identification in cell fusion experiments. Chinese hamster ovary cells with a specific enzyme deficiency resulting in glycine auxotrophy have been fused with human cells. These hybrid cells are being grown by Dr. T. T. Puck (University of Colorado Medical Center, Denver) on selective media to isolate those lines with altered glycine response. Metaphases from the parent hamster line and selected hybrid clones are being studied in our laboratory using the differential staining methods. By banding and analyzing the hybrid cells, it can be determined if specific human chromosomes are present. Analysis of several of these cell clones should permit the assignment of the enzyme in question to a particular chromosome. Linkage studies and gene mapping may follow after the responsible chromosome is positively identified.

Studies on the origins of the cells which repopulate implanted splenic tissue of non-irradiated mice. It has been observed that autotransplantation of thymus, lymph node, and bone marrow at ectopic sites in experimental animals resulted in reorganization (after total necrosis) into structures that were histologically indistinguishable from the original organ. Similar findings were also observed when splenic tissue fragments were ectopically autotransplanted in mice, rats, and rabbits after total splenectomy. These tissues also underwent total necrosis followed by reorganization of the fragments into microscopically similar splenic fragments within eight weeks.

In collaboration with Dr. V. V. Likhite (Harvard Medical Unit, Boston City Hospital) our studies were formulated to elucidate the origin of the cells populating the explanted splenic tissue. To evaluate whether these cells originated from the host or the donor, syngeneic DBA/2 male and female mice were used. After splenectomy of the host, the middle splenic fragment from the animal of the opposite sex was placed in one of the subcutaneous pockets in the left lateral thoraco-axillary region. At various periods after transplantation, splenic implants were found to be from the host. Cultures made from the matrix of the implants exhibited the donor karyotype. In another experiment in collaboration with Dr. G. Brecher (University of California Medical Center, San Francisco), with irradiated hosts, the cells that populated the implant had the donor karyotype. Due to these findings with Dr. Brecher, we wish to use immunocompromised DBA/2 mice in similar autotransplantation experiments.

Cytogenetic studies on two clonal lines of B16 melanoma. Cells from two strains of the B16 melanoma are being studied in cooperation with Dr. G. Sato (University of California, San Diego). The behavior of tumors from each strain varies; one metastasizes extensively while the other does not. Our studies hope to characterize the chromosomal constitution of each strain to determine if karyotypic variations may account for this difference.

Clinical cytogenetic studies on human congenital and developmental disorders. The differential staining methods are being applied in our laboratory in studies of patients with various genetic disorders, abnormalities in gonadal development or function, and selected cases of infertility in the male. Before the use of the new banding procedures in our clinical studies, it was often difficult to precisely identify small variations in chromosome structure. Duplications or deletions of a chromosomal segment, pericentric inversions, and reciprocal translocations can now be more easily recognized. The specific segment or break-point involved can most often be described using the new chromosome nomenclature. The quinacrine mustard procedure is of especial use in studying the Y-chromatin. With this method, Y-chromatin can easily be demonstrated even if it has been translocated to another chromosome. Therefore, we now derive more information about specific chromosome abnormalities from our patient studies.

Pattern of recovery of hemopoiesis in the mouse after irradiation and mechanical depopulation. In cooperation with Dr. G. Brecher (University of California Medical Center, San Francisco) and Dr. W. W. Smith (NCI-LPHY), studies on the hematopoietic differentiative properties of murine spleen implanted in the omenta of irradiated and nonirradiated hosts are currently being published.

Using the AKR-T1Ald marker chromosome system, we have established that donor hemopoietic stem cells fail to survive and proliferate in ectopic sites in normal syngeneic hosts but do so in irradiated hosts. We wished to clarify whether the proliferation in irradiated hosts is due to lack of competition from host stem cells, humoral stimulation from elevated hemopoietins of a minor locus of histoincompatibility between AKR and T1Ald strains. The first two possibilities were explored using T1Ald spleen fragment implants into the omenta of syngeneic AKR hosts which received daily infections of phenylhydrazine in lieu of radiation. This treatment of the host produced an intense hemopoietic stimulus without depletion of the host's own stem cells. Under these conditions, the splenic implants were of host origin. Similarly, host cells were found in spleen and bone marrow grafts from T1Ald or AKR parents into nonirradiated F_1 hybrids. These data confirm the supposition that the proliferation of donor cells in irradiated recipients is made possible by the simultaneous suppression of the host's own stem cells and hemopoietic stimulation due to ablation of the marrow by radiation.

Work on recovery of marrow from mechanically depleted femurs is now being completed. We have demonstrated that hemopoietic repopulation in the depleted femur is invariably of the same composition as the preexisting mix of donor and host cells in that limb, e.g., in chimeras produced by T1Ald marrow transfused into AKR mice after high doses of radiation, repopulation is entirely from donor (T1Ald) type. After local shielding, when a mixture of host and donor hemopoietic cells is established in the shielded limb by post-radiation marrow transfusion, repopulation after mechanical depletion is also of the mixed type. Our initial karyotypic determination of cultured stroma of the repopulating marrow showed that the stroma is of host origin. Presumably, bone is formed from these stromal cells. Karyotypic determination is now being done on cultures from additional animals to extend this finding. If confirmed, it would indicate that there is no evidence for existence of a common stem cell precursor of bone and marrow cells in repopulation after mechanical depletion since the stromal cells are solely of host type even when hemopoietic marrow cells are all donor or mixed.

In a single pilot experiment we have successfully implanted normal human bone marrow into the omenta of heavily irradiated murine recipients. We plan to employ this system to provide an in vivo culture for the study of human stem cell disorders. At the present time we are attempting to discern differences in growth pattern characteristics between normal and leukemic marrow. If this system becomes established, it could serve as an in vivo model for testing chemotherapeutic as well as radiotherapeutic regimen in the treatment of neoplasia.

Sister chromatid exchange in humans. Experiments have been started to study sister chromatid exchanges in chromosomes of somatic cells. This new method involves in vitro replication of cells in the presence of 5-bromodeoxyuriding (BrdU) and exposure to UV light. After staining, the exchanges between the two chromatids of a chromosome can be visualized. The rate of sister chromatid excahnge determined in normal human metaphases can then be compared to the rate found in humans with various disease syndromes or those exposed to potential mutagens. This will permit more detailed studies of chromosome stability in normal and abnormal states.

Keyword Descriptors:

chromosomal banding with differential staining techniques, sister chromatid exchange, human cytogenetics, murine cytogenetics, murine Robertsonian translocation chromosomes, murine spleen transplantation

Publications:

White, B.J., Tjio, J.-H., Van de Water, L.C. and Crandall, C.: Trisomy 19 in the laboratory mouse. I. Frequency in different crosses at specific developmental stages and relationship of trisomy to cleft palate. Cytogenet.Cell Genet. 13: 217-231, 1974.

White, B.J., Tjio, J.-H., Van de Water, L.C. and Crandall, C.: Trisomy 19 in the laboratory mouse. II. Intra-uterine growth and histological studies of trisomics and their normal littermates. Cytogenet.Cell Genet. 13: 232-245, 1974.

White, B.J., Driscoll, E.J., Tjio, J.-H. and Smilack, Z.H.: Chromosomal aberration rates and intravenously given diazepam: A negative study. J.Amer.Med.Assoc. 230: 414-417, 1974.

Tjio, J.-H. and Whang-Peng, J.: Direct Chromosome Preparation of Bone Marrow Cells. In Yunis, J.J. (Ed.): Human Chromosome Methodology. Second Edition. New York, Academic Press, 1974, pp. 157-165.

White, B.J. and Tjio, J.-H.: Translocations Robertsoniennes chez la souris de laboratoire: Consequences sur la genetique et l'evolution. Lyon Medical 233: 197-201, 1975.

White, B.J. and Tjio, J.-H.: AL/N: A homozygous Robertsonian translocation mouse strain identical to TlWh. Cytologia 40 (in press).

Haley, J.E., Tjio, J.-H., Smith, W.W. and Brecher, G.: Hematopoietic differentiative properties of murine spleen implanted in the omenta of irradiated and nonirradiated hosts. Exp.Hematology (in press).

Project No. Z01 AM 21,009-08 LEP
1. Experimental Pathology
2. Cytogenetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cytogenetic Studies

Previous Serial Number: NIAMDD-LEP-13

Principal Investigator: Dr. B. J. White

Other Investigators: Dr. J.-H. Tjio

Cooperating Units: Dr. R. Johnsonbaugh (Pediatric Endocrinology, NNMC, Bethesda)
Dr. E. Driscoll (NIDR-DP)
Dr. J. Sever (NINCDS-PR)
Dr. W. Caveness (NINCDS-LEN)
Dr. M. Sklar (NIAID-LVD)
Dr. D. Symmes (NICHD-BB)
Dr. D. Nebert (NICHD-LBS)

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Cytogenetic and reproductive studies of mice with Robertsonian translocations.

Continued studies of the Rb(5.19)1Wh strain. This strain, developed in our laboratory in 1967, is in generation 32 of inbreeding and has remained normally fertile and stable. It has now been distributed to investigators in Europe and the U.S. for use in a wide variety of studies and continues to be utilized for projects within our own laboratory. A paper reviewing the characteristics of this and other similar strains is currently in press.

Meiotic and other studies of mouse hybrids. During the past year, a long-term study of trisomy for chromosome number 19 was completed. Trisomic newborn and fetal mice were obtained by crossing double translocation heterozygotes, Rb(5.19)1Wh/Rb(9.19)163H, with several other strains. Fetal and placental growth retardation, cleft palate, and degeneration of oocytes were associated with trisomy 19. This study emphasized the similarity between the effects of autosomal trisomy upon growth and development in humans and mice. Two papers describing these findings were recently published. Currently, our laboratory is collaborating with that of Dr. D. Nebert (NICHD-LBS) to study a specific enzyme in trisomic fetuses and their normal littermates. This study of enzyme induction is an attempt to determine the effects of trisomy at a cellular level. Following our description of the method to produce trisomy in the mouse, other investigators have utilized it with other translocation stocks to produce a variety of mouse autosomal trisomy "syndromes", including trisomy for chromosomes 1, 4, 8, 10-12, 15, and 17. Interest in the use of mice as models for studying trisomy-associated malformations is increasing.

Previous reports from this laboratory described reduction of the mouse chromosome number from 40 to 36 by selective crosses of Robertsonian translocation carriers Rb(5.19)1Wh and Rb(6.15)1Ald. The strain with 36 chromosomes has remained normally fertile and stable during the past two years. This strain, along with one of the parent strains, has been exported to Harwell, England, where a variety of studies of murine translocations are in progress. During the past year, our new strain with 34 chromosomes developed by combining Rb(5.19)1Wh, Rb(6.15)1Ald, and Rb(8.17)1IEM has remained fully fertile and has shown no phenotypic or meiotic abnormalities. This new strain was described in a recent publication, which discussed the role of such translocations in evolution and speciation. At present, we are studying the F_1 hybrids of mice with 34 chromosomes. While their fertility is relatively normal, they have frequent meiotic nondisjunction (25% unbalanced spermatocytes). We will be attempting to determine their frequency of trisomic progeny during the coming year. This may help us to determine the viability and characteristics of trisomy for chromosomes 5, 6, 8, 15, and 17 within our stock. So far, our experiments with trisomy 19 suggest that the genetic background of strains used in test crosses strongly influences the frequency, viability, and phenotype of trisomic fetuses; we suspect that this will also be observed in the other trisomy syndromes and hope to investigate this possibility.

Cytogenetic and dermatoglyphic studies of patients.

Twenty-four patients with a variety of genetic or congenital abnormalities were karyotyped using chromosomal banding techniques. These included the following: primary and secondary amenorrhea, Noonan's syndrome, Klienfelter's syndrome, Burkitt's lymphoma with hypogonadism, hypogonadotropic hypogonadism, ambiguous genitalia, idiopathic mental retardation, growth hormone deficiency, Rothmund's syndrome, and Weil-Marchesani syndrome. Variations from normal were found in 25% of the cases (6 patients):

Primary amenorrhea	45,XO (2 cases)
Klinefelter's syndrome	47,XXY/48,XXY,+mar
Idiopathic mental retardation	47,XX,inv(9),+mar
Parent of infant with multiple anomalies	46,XY,t(4;11)
Azospermia	46,XY,21s+

In the past several years, our banding techniques have not demonstrated specific major or minor chromosomal abnormalities in pateints with well documented syndrome complexes. Exceptions to this are our findings in Down's, Klinefelter's, and Turner's syndromes, where an association with major chromosomal defects has been well established by many investigators. Typical results of our ongoing project were the observations of patients with Noonan's syndrome: The phenotype of these individuals resembles that of Turner's syndrome (45,XO chromosome anomaly), although karyotypes prepared with conventional techniques are normal. For this reason, there had been disagreement about whether Noonan's resulted from an occult chromosomal defect (detectable only by banding) or an autosomal dominant gene with variable penetrance. Since we found no chromosomal abnormalities in typical male and female cases using several differential staining techniques (G-, C-, and Q-banding), we favor the latter explanation. Because some patients with this relatively common syndrome are fertile, such findings are of importance in genetic counseling.

The family of the patient listed above with a pericentric inversion of chromosome 9 (47,XX,inv(9),+mar) is currently being studied for the presence of other inversion carriers. Since inversion carriers can now be detected by chromosmal banding techniques and may have an increased risk of having children with unbalanced karyotypes, it is important to detect them for genetic counseling purposes.

Our observations of the relationship of dermatoglyphic features to both clinical and chromosomal findings have been continued as part of a long-term project.

The frequency of sex chromosome defects in prepubertal and teen-aged girls less than third percentile in height in relation to midparental height is being studied in collaboration with Dr. R. Johnsonbaugh (National Naval Medical Center, Pediatric Endocrinology). The height standards charts of Tanner, et al., University of London, are used to select cases. So far, 4 of 13 samples analyzed blindly have revealed abnormalities (2 = 45,XO; 1 = 45,XO/46,XX; 1 = 45,XO/46,X,+mar/47,X,+mar,+mar), suggesting that the population of short girls with parents of average or above average stature has increased frequency of sex chromosome defects.

Cytogenetic studies on the effect of diazepam (Valium) on human chromosomes.

This study of blood samples from healthy young adults given Valium I.V. for oral surgery has been completed. There was no significant chromosomal damage found following a single large dose. The results of this study, done in collaboration with Dr. E. Driscoll (NIDR-CP) have been published.

Karyotypic evaluation of potentially abnormal tissues.

A controlled study of the effects of maternal protein and/or carbohydrate restriciton upon the chromosomes of newborn Rhesus monkeys, in collaboration with Dr. J. Sever (NINCDS-PR), has been completed. The results were negative and have been submitted to the collaborator to combine with data from a variety of other observations made on the Rhesus infants by several other groups.

In collaboration with Dr. Wm. Caveness (NINCDS-LEN), chromosome studies of irradiated Rhesus brain tissue cultures are being done. These results, when combined with those of collaborators doing virus isolation, pathological, and electron microscope studies may clarify the mechanism of endothelial cell proliferation observed after radiation-induced brain damage. In collaboration with Dr. D. Symmes (NICHD-BB), a series of chromosomal inversions in squirrel monkeys is being studied. Fertility, phenotype, and behavioral characteristics of animals from specific matings will be correlated with karyotypic findings. Since inversion heterozygosity in humans may result in offspring with unbalanced karyotypes, these primate studies will provide a model for human defects.

In collaboration with Drs. Sklar and Rowe (NIAID-LVD), the ability of Abelson virus to initiate oncogenic transformation *in vitro* was studied with chromosomally marked virus-treated cultured spleen cells injected into murine hosts. This study of ascites and solid tumors observed in hosts suggested that the virus may initiate oncogenesis *in vitro*. While C-type RNA viruses are known to induce leukemias and lymphomas, oncogenic transformation of lymphoid cells by such a virus *in vitro* had previously not been reported. The results of this completed study have recently been published.

Keyword Descriptors:

mouse Robertsonian trnaslocations and autosomal trisomy syndromes
translocations and inversions in karyotypic evolution
cytogenetics of human syndromes
dermatoglyphics of human syndromes
chromosomal banding with differential staining techniques
Abelson virus-induced murine spleen cell transformation
human chromosomal aberration rates following diazepam
maternal malnutrition and chromosomal aberrations in Rhesus infants
primate brain tissue transformation following X-irradiation

Publications:

White, B.J., Tjio, J.-H., Van de Water, L.C. and Crandall, C.: Trisomy 19 in the laboratory mouse. I. Frequency in different crosses at specific developmental stages and relationship of trisomy to cleft palate. *Cytogenet.Cell Genet.* 13: 217-231, 1974.

White, B.J., Tjio, J.-H., Van de Water, L.C. and Crandall, C.: Trisomy 19 in the laboratory mouse. II. Intra-uterine growth and histological studies of trisomics and their normal littermates. *Cytogenet.Cell Genet.* 13: 232-245, 1974.

White, B.J., Driscoll, E.J., Tjio, J.-H. and Smilack, Z.H.: Chromosomal aberration rates and intravenously given diazepam: A negative study. J.Amer.Med.Assoc. 230: 414-417, 1974.

Sklar, M.D., White, B.J. and Rowe, W.P.: Initiation of oncogenic transformation of mouse lymphocytes in vitro by Abelson leukemia virus. Proc.Nat.Acad.Sci.USA 71: 2077-2081, 1974.

White, B.J. and Tjio, J.-H.: Translocations Robertsoniennes chez la souris de laboratoire: Consequences sur la genetique et l'evolution. Lyon Medical 233: 197-201, 1975.

White, B.J. and Tjio, J.-H.: AL/N: A homozygous Robertsonian translocation mouse strain identical to T1Wh. Cytologia 40 (in press).

ANNUAL REPORT
LABORATORY OF CHEMICAL BIOLOGY
NIAMDD

Much of the research in the Laboratory of Chemical Biology is related to the understanding of relationships between protein structure and function, of the chemical and steric characteristics of antigenic sites in native and denatured proteins, and of the nature of nucleation sites in the folding of polypeptides. The list of proteins under consideration includes sickle hemoglobin, neurophysin and its natural biological ligands, oxytocin and vasopressin, paramecium myoglobin and immobilization antigen, lysozyme, cytochrome c and pancreatic ribonuclease. Staphylococcal nuclease continues to be used as a model protein for various studies of the kinetics of folding and unfolding. The work on isolation and characterization of human interferon is being carried on and promising results have been obtained on the use of a lymphoblastoid cell line which can be grown in liquid culture with stirring, and which produces respectable amounts of interferon when challenged with Sendai virus.

I. Three-dimensional protein structure and its relationship to amino acid sequence.

A. Complementation of protein fragments.

Information of value in the investigation of polypeptide folding can be obtained by a study of the cooperative interaction between two or more fragments of a protein molecule. This approach to the formation and stabilization of tertiary structure was first used by Richards in his work on the RNase-S system. In this laboratory the same kind of approach has been used with staphylococcal nuclease and ribonuclease, and more recently with cytochrome *c*, lysozyme and hemoglobin. It was reported in the annual report for 1973-1974 that two fragments of staphylococcal nuclease, one containing residues 50-149 and a second containing residues 1-126 can combine very rapidly to form two alternative complexes, both possessing enzymatic activity and exhibiting many of the physical properties of native nuclease. The kinetics and thermodynamics of these two complementary systems has now been worked out with greater precision. The results show that both types of complex are formed initially in approximately equal amounts but that Type I predominates in a ratio of approximately 3 to 1 at equilibrium at 24°. The ratio of the two types of complementation is approximately 2 to 1 at 6°. Formation of both types of complementation involves non-covalent interaction of the fragments, with redundant portions of the chains protruding in a random form as "polypeptide tails." In Type I the complex consists of residues 1-48 interacting with residues 50-149. In Type II the interaction involves all of fragment 1-126 and the (111-149) portion of fragment 50-149. The two complex types may be stabilized by the addition of the ligands, Ca^{++} and thymidine diphosphate (pdTp) and the redundant portion of the sequence may then be removed by trypsin digestion. A greater thermodynamic stability is indicated for the Type I complementation, by its predominance at equilibrium. Type I has been crystallized and efforts are now being made to crystalize Type II as well. X-ray crystallographic studies will be initiated in the next few months and the results obtained will make it possible to relate the relative stabilities of the two forms to specific interactions between residues in the two structures.

In the course of preparing the interacting fragments mentioned above, evidence for a precursor of nuclease has been obtained. This precursor appears to consist of the usual nuclease sequence plus 18 additional amino acid residues at the amino terminal end. The exact structure of the additional portion and its influence, if any, on nuclease function are currently under investigation. (Taniuchi, Parker, Davis, DiBello)

Similar studies designed to give information about the formation of stable solution structures are being carried on with cytochrome c and egg white lysozyme. A heme-containing fragment of horse heart cytochrome c, residues 1-65, has been isolated from a cyanogen bromide digest of the native protein. As with the case of fragments of staphylococcal nuclease, the complex formed by the fragment and native cytochrome c (residues 1-104) demonstrates resistance to tryptic digestion and it appears that, here too, redundant overlapping stretches of sequence may be removed by proteolysis with retention of many of the spectral properties of cytochrome c itself. Antisera are being prepared against portions of the sequence for use as tools for discriminating between native and denatured states of the polypeptide chains. (Hantgan and Taniuchi)

It has been shown previously that fully reduced lysozyme, containing 8 SH groups, will refold to yield, at equilibrium, nearly quantitative conversion to the native structure containing the correct 4 SS bonds. The course of this conversion is being studied by the isolation of intermediate forms by gel filtration, and by the identification of the particular half-cystine residues that participate in each SS bond formation. The mixture of products formed early in the reoxidation of the reduced chain contains the native structure to the extent of 40-50%. Subsequent conversion of the remainder from inactive SH-free forms to the native form involves disulfide interchange. (Acharya and Taniuchi)

In a continuation of efforts to prepare semisynthetic staphylococcal nuclease for crystallographic studies, several large scale syntheses of the fragment containing residues 6-48 have been carried through using the solid phase Merrifield method. Work is progressing on the purification of the synthetic polypeptide by complexing with the natural fragment (49,50-149), followed by digestion of the "incorrect" synthetic sequence with trypsin in the presence of pdTp and calcium ions. Techniques have been perfected, in the meantime, for the preparation of crystals of the complex, suitable for X-ray analysis using the two components prepared from native nuclease. It is already known that this complex is isomorphous with undegraded nuclease. Success in the crystallographic studies of the semisynthetic complex will give direct evidence of the utilizability of the solid phase method for preparing large molecules in error-less form (Vigna and Taniuchi)

B. Immunochemical studies of protein conformation.

Previous reports have discussed the use of antibodies for the study of protein antigen conformation. In general, antibodies to staphylococcal nuclease inactivate the enzyme upon formation of the complex. It has been possible to prepare non-precipitating monospecific antibodies to various portions of the nuclease structure. This was accomplished by passing the antinuclease gammaglobulin through an affinity column prepared by attaching fragments of the native protein to Sepharose. Polypeptide fragments of the protein appear to be involved in equilibrium between a random uncoiled state and the "native format." The latter term relates to the three-dimensional form which characterizes the particular fragment when it is part of intact native nuclease molecule. A fraction of the antisera specific to the sequence 99-149 was bound to a Sepharose column bearing the 99-149 sequence. This anti-fragment (99-149) was further separated into anti-(99-126) and anti-(127-149) by absorption on a (127-149)-Sepharose immunoabsorbent column. Antisera such as those described above recognize the native formats of protein antigens in contrast to antibodies produced by injection of the more random fragments of the nuclease sequence which do not cause inactivation when added to nuclease assays.

Current studies involving synthetic antigens have extended the use of antibodies for studies of conformation. Thus, the sequence (53-69) of nuclease has been synthesized and attached to activated Sepharose through its terminal amino group. This synthetic absorbant has a strong affinity for the antibody population that recognizes the native format of the normally α-helical region 53-69. The bound antibody, after displacement from the column by excess peptide, is operationally monospecific and inactivates nuclease.

The use of antibodies in the above fashion serves as an important tool for the study of protein conformation, in general, since it permits the mapping of the exterior of the globular molecules and makes it possible to localize individual antigenic sites by this combination of organic synthesis and affinity chromatography. (Curd, Gerlt and Schechter)

In a collaborative study with Sachs and Shearer of the Immunology Branch, NCI, Berzofsky and Schechter have continued studies of the immune response genes (IR genes) which map within the major histocompatibility complex (H-2) of mice. These studies, now in progress, are concerned with the relative proportions of antibodies to different antigenic determinants on the nuclease molecule that are made under IR gene control by high responder and low responder strains. Further fractionation of antisera can then be performed using affinity columns bearing selected portions of the polypeptide backbone of nuclease. Early results indicate that both of the high responder strains studied so far produce about the same fraction of antinuclease antibodies (10%) directed against the 99-149 portion of the nuclease sequence.

C. Studies on the conformation of hemoglobin A and hemoglobin S.

The approaches, described above, for the immunochemical study of the conformation of nuclease, have been extended to the study of normal and mutant hemoglobins. A peptide containing the amino terminal 55 amino acids of the β-chain of hemoglobin-S was prepared and used to elicit antibodies in goats. A radioimmunoassay was developed using a radioiodinated derivative of the 55-residue fragment together with dextran-coated charcoal. The complex formed between the specific antibody population and the radioiodinated fragments had a measured affinity constant of 10^8 M^{-1}. The radioiodinated fragment may be displaced by the β-chain of hemoglobin-S and, to a smaller extent, of hemoglobin-A. The antibodies elicited by the 55-residue fragment may be further separated to yield a subpopulation much more specific for antigenic determinants on the 55-residue S-fragment and for S-globin than for corresponding "A" determinants. Such antibody preparations may be useful as immunological reagents in diagnosis and screening. (Curd, Young and Schechter)

Antibodies have also been prepared to both intact hemoglobin A and S molecules. The specificity of these antibodies for their corresponding types of hemoglobin has been enhanced by batch immunoadsorption, fractionation on Sepharose-hemoglobin and on Sepharose-peptide columns. Specificity has been established, and the strength of the interactions with hemoglobin have been determined, using a radio-immunoassay employing radioactive carbamylated hemoglobin and a double antibody method.

These methods have allowed the preparation of antibodies with very high specificity for hemoglobin S which may eventually be useful as screening reagents. More immediately, however, they are being used to examine the effects on hemoglobin conformation of various ligands (O_2, CN, diphosphoglyceic acid (DPG)) and to examine the nature of the antigenic determinants in this region of hemoglobin S and A. (Young and Schechter)

The availability of several fragments from the hemoglobin molecule - some prepared by proteolytic digestion and some prepared by Merrifield solid phase synthesis - has led to a study of the interaction of these peptides with hemoglobin. It is believed that some peptides may be found that will inhibit gelation of hemoglobin S and thus be useful in studying the nature of the tetramer contacts and possibly also in designing inhibitors of sickling. These peptides have been screened by a variety of means, including the minimum gelling assay, the kinetic gelling assay of Eaton and Hofrichter, equilibrium dialysis and affinity chromatography. To date the results have been complicated by deviations from physico-chemical ideality but methods to overcome these problems are being developed. (Young, Curd, Eastlake, and Schechter)

Experiments have been carried out to determine the level of denaturation, if any, when a protein antigen is emulsified with complete Freund's adjuvant. The sensitive technique of electron paramagnetic resonance was applied to various forms of hemoglobin. This technique for detecting changes in the paramagnetic iron atoms indicated that there was no significant denaturation of the protein. (Berzofsky and Schechter)

D. Study of protein conformation and biological function with semisynthetic peptide-protein complexes.

Semisynthetic RNase-S derivatives have been prepared with a leucine or glycine residue replacing the phenylalanine residue normally present at position 8. Resulting complexes with RNase-S-protein were active in spite of the introduction of these helix-breaking residues in a portion of the chain that is normally helical. An enzymically inactive derivative with 4-fluorohistidine at position 12 has been synthesized. This histidine has also been replaced by an analog containing carbon 13. The influences of such substitutions can be studied in detail by nuclear magnetic resonance and the results should be of value in understanding the role of specific amino acids in the stabilization of the RNase-S complex. (Chaiken, Dunn, Pandin and Taylor)

II. Studies on neurophysin

Several studies are under way on the structural and functional aspects of neurophysin and its complexes with oxytocin and vasopressin. Equilibrium dialysis experiments have shown that the vasopressin analogue, methionyl-tyrosyl-[^{14}C]phenylalanineamide, interacts with each of bovine neurophysins I and II specifically, having characteristics of binding similar to, but distinguishable in important ways, from those of the intact peptide hormones. The availability of the analogue peptide has allowed the preparation of an affinity matrix, to be used in future studies of neurophysin-hormone complexes.

Neurophysins were found to be highly susceptible to inactivation by disulfide interchange. Combined with the observation of stabilization of interchange by the ligand Met-Tyr-Phe amide, the results are consistent with the hypothesis that the neurophysins are biosynthesized as part of a larger precursor protein and that part of the additional polypeptide of this precursor may correspond to sequences analogous to those of the neurohypophyseal peptide hormones.

In parallel with these studies, a strongly antigenic conjugate of bovine neurophysin with poly Ala-poly Lys has been prepared. The resulting conjugate has been used to elicit antibodies in rabbits. Such antibodies will be of value in a search, by affinity chromatography, for a possible larger precursor of neurophysin. Finally, efforts are underway to isolate and purify neurophysin from human posterior pituitary glands. A comparison of the human hormone with the bovine will furnish evidence about the essentiality of various parts of this hormone molecule. (McCormick, Taylor, Chaiken and Randolph)

III Isolation and characterization of proteins of Paramecium.

Following up on a long-term interest in proteins of the convenient monocellular organism, Paramecium, work has continued on a myoglobin-like moiety and on the so-called immunobilization antigen present as a surface component of the cell. Highly purified myoglobin yields 5 heme-containing bands upon disc-gel electrophoresis. All of these have molecular weights of about 16,000 and the isoelectric points of the 5 have been determined to range from 5.54 to 4.16. As an contribution to the understanding of evolutionare relationships, a full chemical characterization of this myoglobin system is underway.

The immobilization antigen has been highly purified for study of the quaternary structure. The subunit nature of this large, fibrous protein is under study by a variety of physico-chemical methods. (Steers, Davis and Lee)

IV Methodology

A. Quantitative affinity chromatography.

Further study of the use of affinity chromatography for quantitative evaluation of ligand binding has proceeded. When a ligand is bound to Sepharose, a column prepared from such material will bind the corresponding protein molecules. The strength of this binding can be estimated quantitatively by determining the degree to which the free ligand, or an analog, displaces the protein from the solid phase support. The technique has been used, for example, in a study of the affinity of RNase-S-protein for several of the synthetic peptides described above in section II. The procedure has promise for a number of other systems, such as the neurophysin-vasopressin complex mentioned above. (Taylor, Dunn and Chaiken)

B. Nuclear magnetic resonance studies of structure and function in S. nuclease.

The study of complexes of staphylococcal nuclease with the substrate analog, pdTp, and alkaline earth metal ions has been carried out using lanthanides, such as Gd(III). The introduction of such paramagnetic ions in place of the normal calcium ion makes it possible to determine distances between side-chain groupings in the protein and in the substrate analog due to the perturbance of NMR resonances by the metal ion. These studies have given direct evidence demonstrating that the geometry existing in solution is very similar to that seen by crystallographic analyses. (Schechter (and Feldmann, DCRT))

V Interaction between transfer RNA and proteins.

The complementation of tRNA molecules with their corresponding synthetases can be studied by immunological methods. Although rabbits and mice produce only small amounts of low affinity antibodies to tRNA molecules, a high affinity binding activity has been found in NZB/NZW mice which exhibit an autoimmune disease very similar to systemic lupus erythematosis (SLE) in humans. Such tRNA binding activity was also found in some SLE patients.

tRNA specific for leucine was investigated before and after bacteriophage infection of E. coli cells. The amino acid acceptance of tRNA-Leu seemed to remain unaltered, but the isoacceptor tRNA distribution has been drastically changed. Leu-$tRNA_{II}^{Leu}$ which is present in negligible amounts in the uninfected cells becomes the major tRNA after infection. In addition, two chromatographically distinct new peaks of Leu-tRNA appear after infection. The dramatic increase in Leu-$tRNA_{II}^{Leu}$ is compatible with the availability of its assigned codons in the MS2 message for the coat protein.

The molecular weight of the homogeneous phenylalanine Salmonella tRNA synthetase has been established by disc gel and ultracentrifugation methods as 220,000 with an $\alpha_2 \beta_2$ subunit structure, with subunits of 86,000 and 36,000 molecular weight. The amino acid composition and various spectroscopic and immunochemical properties of the enzyme have been established. This complements paralleled work on the histidine synthetase.

The location of the fluorescent group on the phenylalanine tRNA has been established as the "X-base" of the variable stem and this offers a new means of studying the conformation and interactions of tRNA with a specific probe on a region not accessible before. The interaction of this labeled tRNA with the synthetase is being studied. In addition a hexapeptide which is expected to be complimentary to the tRNA has been synthesized, with a radioactive label, and is being purified for binding studies.

The tRNA-antibody project may lead to new information on the origin, development, and genetics of certain autoimmune diseases, particularly Systemic Lupus Erythematosus. the tRNA regulation project will provide a better understanding of the cellular mechanisms following viral infection.

The development of antibodies to tRNA, and other nucleic acids, offers great potential for studying the conformation of nucleic acids (by methods analogous to those we have used for studying the conformation of proteins) and for probing various questions related to the structure and function of nucleic acids in the ribosome, nucleus and in several biological processes. The characterization of these NZB/NZW mice antibody proteins is a start in this direction.

The analysis of the interaction of several tRNA molecules with their cognate synthetase and with specific peptides will be used in the study of protein-nucleic acid interactions by physico-chemical techniques and may give information on the mechanisms of these biologically important phenomena. (Eilat, Di Natale, Thomas, Schiller and Schechter)

VI Purification and structure determinations of human interferon.

The techniques for the purification of human interferon from tissue culture fluids have been further elaborated. Human fibroblasts, buffy coat cells and lymphoblast cells challenged with either Sendai or poly I-poly C produce interferon activity which in all cases studied exhibits a molecular weight of approximately 20-25,000 and may be adsorbed to an affinity column of Sepharose bearing purified sheep antibodies to leucocyte interferon. The active material bound to such columns is then further purified on Sepharose G-100 gel filtration columns and by isoelectric focusing in sucrose density gradients. By adding radioactive amino acids to the tissue cultures during interferon production quite high levels of radioactivity may be incorporated into the fractions possessing interferon activity. The resulting radioactive fraction, which exhibits an isoionic point of about 6.2, can then be submitted to automated Edmund sequencing in the presence of a carrier protein such as apomyoglobin. The release of radioactive phenylthiohydantoins may be monitored by high pressure liquid chromatography in the presence of carrier ^{12}C phenylthiohydantoins. Some preliminary results on the identity of residues in 5 of the first 12 positions in the protein chain have thus been obtained. The results are, however, uncertain because of current inability to rule out the presence of a major impurity.

Interferon, which is known to be a glycoprotein on the basis of experiments by Weil _et al_. in Austria, was produced in culture in the presence of highly radioactive glucosamine. After purification of such carbohydrate-labelled material, biological activity was followed during extensive degradation with a spectrum of carbohydrases which removed 85-90% of the label. This occurred without loss of the viral activity.

Studies are underway to determine optimal conditions for interferon production in the convenient lymphoblastoid cell system. (Bose, Corley, Gurari-Rotman, Ruegg and Anfinsen)

Project No. Z01 AM 25,000-02 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation, structural determination, and synthesis of human interferon

Previous Serial Number: NIAMDD-LCB-8

Co-investigators: Christian B. Anfinsen
Sikta Bose
Lila Corley
Dalia Gurari-Rotman
Urs Ruegg

Man Years:
Total: 4.5
Professional: 4.5

Project Description:

Objectives: Eventual synthesis of human interferon would appear to be the only practical approach to the thorough clinical trial of this antiviral substance since it is present in such minute amounts in biological sources. The end result of this study can only be achieved through isolation of sufficient amounts for structure determination, and for the study of the possibility that parts of the molecule can be removed by chemical or enzymic degradation without loss of functional capacity.

Methods Employed and Major Findings: Leucocyte, fibroblast and lymphoblastoid interferons are removed from crude tissue culture fluids by columns of anti-leucocyte interferon attached to Sepharose-4B. The antibody was prepared in sheep using, as antigen, material that had been partially purified by gel filtration through Sephadex-G100 columns. Anti-interferon in the γ-globulin fraction was purified before attachment to Sepharose by passage through an affinity column bearing the protein impurities that might be present in the crude IF-containing culture fluid (serum proteins and proteins of the buffy coat cells, allantoic fluid, and Sendai virus). Many of the impurities in the crude fibroblast and lymphoblastoid interferon were presumably not recognized by the sheep antibodies induced by leucocyte interferon and these were much more effectively purified as the result of this "common denominator" approach. Interferon could be harvested efficiently from the crude starting materials when a carrier protein (bovine serum albumin and, later, cytochrome c) was added to the eluting buffers to counteract losses, presumably due to adsorption on purification and assay equipment. All these varieties of interferon exhibit molecular weights of approximately 20,000-25,000.

Since the amounts of interferon that we are likely to obtain, even with unusually good recovery, will be very small, we have resorted to an approach involving radioactivity. Human fibroblast, buffy coat cell, and lymphoblastoid cell cultures, induced with poly I - poly C or Sendai virus, have been incubated in the presence of highly radioactive amino acids. Any given incubation involved only two or three tritium labelled radioactive amino acids. Following purification by affinity chromatography, gel filtration, and disc gel electrophoresis or electrofocusing, the minute amounts of radioactive interferon are placed in an automatic Edman sequencing apparatus together with apomyoglobin as a carrier protein. At any step of degradation in which radioactive counts are removed from the protein chain being degraded, the phenylthiohydantoin may be identified by thin layer chromatography.

By such methods, purified samples of interferon have given valine residues in positions 1 and 10, lysine in position 8 and alanine in positions 11 and 12. Until direct information about the purity of the material being subjected to sequence analysis is obtained, these identifications could be spurious and attributable to impurity present in much larger amounts of interferon itself. The current experiments using lymphoblastoid cells should simplify and expedite the research since these cells may be grown and subcultured much like bacteria and produce fairly high levels of interferon in a reproducible way.

The carbohydrate components of interferon have been studied using radioactive glutamine incorporation to label the sugar moieties. Digestion of such radioactive interferon with a broad spectrum of glycosiases removes 85-90% of the labelled sugar without loss of antiviral activity. At the same time the interferon loses much of its heterogeneity, presumably due to variable contents of sialic acid, and yields one fairly sharp zone upon electrofocusing.

This work will be continued with efforts to improve on purity and the use of all the 20 amino acids in radioactive form.

The early phases of our work on interferon have been summarized in PNAS 71 3139, 1974.

Keyword Descriptors:

interferon, tissue culture, affinity columns, structure studies

Honors and Awards: None

Publications:

Anfinsen, C.B., Bose, S., Corley, L., and Gurari-Rotman, D: Partial purification of human interferon by affinity chromatography. Proc. Nat. Acad. Sci. U.S.A. 71: 3139-3142, 1974.

Project No. Z01 AM 25,001-01 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation and characterization of the immobilization antigen from paramecium.

Previous Serial Number: none

Principal Investigators: Edward Steers, Jr.
Richard H. Davis, Jr.

Man Years:

Total: 0.9
Professional: 0.6
Other: 0.3

Project Description:

Objectives: To purify and determine the quaternary structure of the immobilization (surface) antigens from paramecium.

Methods Employed: Paramecium are grown and harvested as previously described (Serial No. NIAMDD-LCB-13). The cell debris after homogenation and centrifugation is treated by the method of Preer for whole cells. The 0-50% ammonium sulfate precipitate is dialized against 0.05M phosphate pH7.3 buffer. The pH of this dialized solution is lowered to 2-2.5 with HCl. The resultant precipitate is discarded and the pH of the solution increased to neutrality. The antigen is further purified by fractionation with ammonium sulfate (0-50%). After dialysis, this fraction is applied to a Sephadex G200 column and the elution monitored at 280nm. The purification is followed immunologically.

Significance to Biomedical Research: The immunologic specificity of the protozoan Paramecium has been shown to be the result of a surface associated protein (immobilization antigen) whose antigenic specificity is under the control of cytoplasmic and nuclear determinants.

Although researchers agree that the protein responsible for the immobilizing reaction in paramecium is a large, fiberous protein of approximately 300,000 daltons, there is conflicting evidence concerning the existance and number of subunits composing the protein. The existence or lack of subunit structure will give insight on the gene(s) responsible for their production and aid in understanding the cross-reactions previously observed in gel diffusion test and the method(s) by which this protein is regulated.

Methods of purification currently being developed with the extraction and purification of myoglobin from paramecium also.

Proposed Course of Project: Following purification, the quaternary structure of the immobilization antigen will be examined by various physicochemical techniques.

Keyword Descriptors:

Protozoa, paramecium, antigen, immobilization antigen, quaternary structure, subunits

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,002-02 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974, through June 30, 1975

Project Title: Isolation and characterization of myoglobin from paramecium.

Previous Serial Number: NIAMDD-LCB-10

Principal Investigator: Edward Steers, Jr.
Richard H. Davis, Jr.

Man Years:

Total: 2.1
Professional: 1.4
Other: 0.7

Project Description:

Objectives: To purify the heme containing protein in Paramecium and characterize it fully including sequential analysis.

Methods: Paramecia are grown on grass extract and Aerobacter cloacae in 5 gallon carboys (Steers, E., Jr., PNAS 48: 867, 1962). The cells are harvested by passage through a commercial cream separator, followed by centrifugation in a clinical centrifuge. The packed cells are resuspended in approximately two (2) volumes of 0.05M phosphate buffer pH7.3 containing 0.2M raffiuose and 1M NaCl to prevent lysis of mitochondria. This suspension is then homogenized in a Dounce tissue homogenizer at 0°. The lysed cells are centrifuged and the pink supernatent retained. The 50-75% ammonium sulfate precipitated fraction of this supernatent is chromatographed on a Sephadex G75 column. The column effluent was monitored at 416nm and by acrylamide disc gel electrophoresis using the heme specific stain Benzidine-HCl or Amido Swartz. After pooling and concentrating (75% ammonium sulfate) the appropriate fractions, the pigment is further fractionated by chromatography on Sephadex G50 and the effluent monitored as already described. The appropriate fractions are pooled and concentrated with ammonium sulfate at 75% saturation.

The precipitate after dialysis against .05M phosphate buffer, pH7.3, is electrophoresed on 7.5% acrylamide gels and/or electrofocused on 5% acrylamide gels with pH4-6 or 3.5-10 ampholites. The appropriate zones are eluted from the acrylamide gels in phosphate buffer or collected by drop-wise elution depending upon the electrophoretic technique employed.

Major Findings: Acrylamide disc gel electrophoresis revealed five (5) heme-containing bands. The two major forms comprise 65% of the total and the three (3) minor forms the remaining 35%. All five components have been found to weigh approximately 16,000 daltons. A correlation between the individual bands and their respective isoelectric points was accomplished by cutting the respective zones from standard 7.5% gels and electrofocusing them directly on 5% acrylamide disc gels. The isoelectric points of the five (5) electrophoretic components determined in this way were found to be 5.54, 5.18, 4.98, 4.51 and 4.16 respectively.

The physiological role of this myoglobin in paramecium continues to remain obscure as similar extraction techniques with a closely related protozoan, tetrahymena, showed no such pigment to be present. Whether the electrophoretic forms ms show func-tional heterogeneity or are functionally homogeneous is also not known at the present time.

Significance to Biomedical Research: Many invertebrate hemoglobins have been identified in a variety of sources. This is the first instance of a molecule of this class appearing in the protozoa. Of especial interest is the possible role of this molecule which may be quite different than the role ascribed to these pigments in higher organisms as well as the apparent structural difference where the heme group appears to be covalently bound to the protein. While the spectral and functional characteristics of the molecule are clearly those of the hemoglobin class of compounds, the covalent attachment of the heme is characteristic of the cytochromes.

Proposed Course of Project: Following the successful purification of Paramecium hemoglobin, a full characterization is anticipated including sequence analysis. Procedures are currently being developed for separation and sequential analysis of select peptides for each of the myoglobin species isolated.

Keyword Descriptors:

heme, paramecium, myoglobin, hemoglobin, protozoa.

Honors and Awards: None

Publications:

Davis, R.H., Jr. and Steers, E., Jr., Multiple forms of myoglobin from *Paramecium aurelia*. J. Comp. Biochem. and Physiol., in press.

Project No. Z01 AM 25,003-05 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of protein conformation and biological function with semisynthetic noncovalent peptide-protein complexes.

Previous Serial Number: NIAMDD-LCB-4
NIAMDD-LCB-5

Principal Investigators: Irwin M. Chaiken
Ben M. Dunn
Mario Pandin

Other Investigator: Hope C. Taylor

Cooperating Units:
1) Physical Sciences Laboratory, Laboratory of Chemical Physics, NIAMDD
2) Laboratory of Chemistry, NIAMDD (Louis A Cohen and Kenneth L. Kirk)
3) Laboratory of Chemistry, NHLI (Edward A Sokoloski)
4) National Institute of Child Health and Development (Jack S. Cohen)

Man Years:
Total: 0.7
Professional: 0.6

Project Description:

Objectives:
1) To define general approaches for the use of semisynthesis in the study of interacting peptide-protein systems.
2) To prepare, by chemical synthesis, polypeptide and protein fragments which interact with complementary native proteins or protein fragments to form biologically active complexes.
3) To purify semisynthetic derivatives in a state suitable for detailed conformational and functional characterization.
4) To examine the influence of side-chain characters on peptide-protein interaction, conformational stability, and function.

Methods Employed; A specific protein fragment, ribonuclease-S-(1-20) has been studied with respect to the noncovalent, enzymically active ribonuclease-S complex formed upon addition to the complementary fragment ribonuclease-S-(21-124). Semisynthetic analogues of the native complex have been obtained by chemical synthesis (solid-phase procedure) of ribonuclease-S-(1-20) variants. With the latter, the microenvironments of specifically chosen loci have been studied, using spectroscopic (carbon 13 and proton NMR, absorption spectroscopy) and enzymic methods.

Studies also have been pursued for the bovine neurophysin II - oxytocin complex, by synthesis of oxytocin derivatives.

Major Findings: The general utility of selective protein modification by semisynthesis has been reinforced and extended.

Analogues of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-20) fragment. Analogues for the α-helical position 9 have revealed critical correlations between the experimentally observed propensity for α-helix formation and empirical propensity parameters (generated by several groups by examination of proteins of known sequence and conformation). Additionally, analogues at the active site His 12 position have been made containing carbon 13 and fluorine 19 atoms. Such analogues have helped refine the view of the participation of His 12 in ribonuclease catalysis and, in the case of the enzymically inactive 4-F-His 12 derivative, have provided a vehicle for future studies of the binding of substrates at the active site.

Study of the interaction of bovine neurophysin II with ^{13}C-enriched synthetic oxytocin allowed implications to be made on the mode of interaction of the neurohypophyseal hormone with its carrier protein.

Significance to Biomedical Research: The above studies provide insight into the potential use of semisynthesis in studying and designing peptide-protein systems of biomedical (including therapeutic) interest. The specific results of this work should allow a more complete understanding of the structural bases for conformation and biological function of protein-containing systems in general.

Proposed Course of Action:

1) Crystallization of semisynthetic Ribonuclease-S'.
2) The study of several of the ribonuclease-S analogues already prepared, such as [4-F-His^{12}]Semisynthetic ribonuclease-S', both in the crystal form and in solution.
3) The design of further derivatives of semisynthetic noncovalent peptide-protein complexes for the study other loci, in a program aimed at eventually defining general rules of amino acid function.

Keyword Descriptors:

semisynthesis, solid phase peptide synthesis, carbon 13 NMR, proton NMR, ribonuclease-S', semisynthetic ribonuclease-S', neurophysin II-synthetic oxytocin complex.

Honors and Awards: None

Publications:

Dunn, B.M., Di Bello, C., Kirk, K.L., Cohen, L.A., and Chaiken, I.M.: Synthesis, purification, and properties of a semisynthetic ribonuclease-S incorporating 4-fluoro-L-histidine at position 12. J. Biol. Chem. 249: 6295, 1974.

Chaiken, I.M., Cohen, J.S., and Sokoloski, E.A.: Microenvironment of histidine 12 in ribonuclease-S as detected by ^{13}C nuclear magnetic resonance. J. Am. Chem. Soc. 96: 4703, 1974.

Chaiken, I.M., and Dunn, B.M.: Semisynthetic ^{13}C-enriched and ^{19}F-labelled peptide-protein noncovalent complexes. Proc. 13th Euro. Pept. Symp. (1975, in press).

Dunn, B.M., and Chaiken, I.M.: Relationship between α-helical propensity and formation of the ribonuclease-S-complex. J. Mol. Biol. (1975, in press).

Project No. Z01 AM 25,004-02 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Quantitative affinity chromatography

Previous Serial Number: NIAMDD-LCB-7

Principal Investigators: Hope C. Taylor
Ben M. Dunn
Irwin M. Chaiken

Man Years:
Total: 0.8
Senior Professional: 0.3
Other: 0.5

Project Description:

Objectives:
1) To continue development of methods using affinity chromatography to determine ligand binding constants.
2) To expand this method to successively more complex protein systems of biological interest.
3) To more deeply understand general principles of affinity chromatography of macromolecules.

Methods Employed: Chromatography of highly purified staphylococcal nuclease was performed on columns of deoxythymidine-5'-phosphate-3-(p-aminophenyl) phosphate-Sepharose, in the presence of various concentrations of competing active site ligand, deoxythymidine-3',5'-diphosphate. Elution behavior on the above affinity matrix, and others prepared by dilution with unsubstituted Sepharose 4B, has been compared to behavior predicted by equations derived from first principles.

More recently, highly purified bovine pancreatic ribonuclease has been chromatographed on an affinity matrix of uridine-3'-phosphate-2'-(p-aminophenyl)phosphate-Sepharose in the presence of varying cytidine-2'-phosphate concentration, as well as varying protein concentration and ionic strength. Its elution behavior and that of ribonuclease derivatives, including Ribonuclease-S, [p-F-phe^{8}1-15 peptide] SRNase-S', and [4-F-His^{12}1-15 peptide] SRNase-S', have been compared to the derived equations as well as our hypotheses about non-specific interactions and predictions of other researchers.

Major Findings: In each case, the soluble ligand has been shown to effectively compete with matrix bound ligand. Where enough material was available to run several columns (Nase, RNase-A and RNase-S), this affinity disruption by soluble ligand was shown to result in enzyme elution at a position inversely dependent on ligand concentration and directly so on its binding constant (K_d, as determined by other methods) with the competing ligand. The determined elution volume, when used in derived equations, permits calculation of binding constants for both soluble and matrix bound ligand to the protein. Further, given a single affinity matrix, the binding constants of several ribonuclease derivatives could be determined.

Significance to Biomedical Research: Application of affinity chromatographic methods will readily facilitate determination of a binding constant for any ligand, or possibly significant ligand derivative, to any protein or derivatized protein. It would provide an alternative to often imprecise or impracticable bioassays for hormone (or other ligand) binding to protein. Studies of the interaction between peptide derivatives and complementary protein fragments could yield important information about the effect on structural integrity, hence, on biological activity, of biologically altered enzymes and binding proteins.

Proposed Course of Project:

1) Further binding constant determinations for different protein semi-synthetic derivatives and inhibitors in the bovine pancreatic ribonuclease system.

2) Extended application to other cases including important peptide-protein and protein-protein systems.

Keyword Descriptors:

affinity chromatography; staphylococcal nuclease; bovine pancreatic ribonuclease; Sepharose 4B; ligand binding; semi-synthetic ribonuclease-S complexes; matrix bound nucleotide; inhibitor binding constants.

Honors and Awards: None

Publications:

Dunn, B.M., Chaiken, I.M.: Quantitative affinity chromatography. Determination of binding constants by elution with competitive inhibitors. Proc. Nat. Acad. Sci. U.S.A. 71: 2382-2385, 1974.

Dunn, B.M. and Chaiken, I.M.: Evaluation of quantitative affinity chromatography by comparison with kinetic and equilibrium dialysis methods for the analysis of nucleotide binding to staphylococcal nuclease. Biochemistry (1975, in press).

Project No. Z01 AM 25,005-02 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Functional and physicochemical characterization of bovine neurophysin-neurohypophyseal hormone complexes

Previous Serial Number: NIAMDD-LCB-3

Principal Investigators: Irwin M. Chaiken
Raymond E. Randolph
Hope C. Taylor

Man Years:
Total: 0.8
Professional: 0.3
Other: 0.5

Project Description:

Objectives:

1) To study the characteristics of binding of bovine neurophysins to the neurohypophyseal hormones, oxytocin and vasopressin.

2) To characterize the aggregation properties of the neurophysins, with respect to the role of such aggregation in hormone-neurophysin interaction.

3) To describe possible biosynthetic interrelationships between the neurophysins and the neurohypophyseal peptide hormones.

Methods Employed: Binding of ^{14}C-labelled peptide ligands to bovine neurophysins I and II has been studied by equilibrium dialysis. Competition of binding by unlabelled peptide hormone analogues also was studied. The possibility of doing studies on insoluble affinity matrices is being explored.

Ultracentrifuge studies of the neurophysins in the presence and absence of hormone or peptide analogues have been carried out using sedimentation-equilibrium methods.

The stability of the neurophysins has been examined with respect to its ability to withstand disulfide interchange, again alone or with peptide ligands present.

Major Findings: Equilibrium dialysis experiments have shown that the vasopressin analogue, methionyl-tyrosyl-[^{14}C]phenylalaninamide, interacts with each of bovine neurophysins I and II specifically, having characteristics of binding similar to, but distinguished in important ways from, those of the intact peptide hormones. The analogue peptide serves as a valuable model and has allowed the preparation of an affinity matrix, to be used in future studies of neurophysin-hormone interacting complexes.

Ligands affect aggregation properties, in such a way as to suggest a relationship between the state of aggregation of the neurophysins and the quantitative character of the interaction with hormones.

Neurophysins were found to be highly susceptible to inactivation by disulfide interchange. Combined with the observation of reduction of this susceptibility by the ligand Met-Tyr-Phe amide, the results are consistent, among other possibilities, with the he hypothesis that the neurophysins are biosynthesized as part of a larger precursor protein and that part of the additional polypeptide of this precursor may correspond to sequences analogous to those of the neurohypophyseal peptide hormones.

Significance to Biomedical Research: The results so far obtained suggest aspects of neurohypophyseal hormone biology and chemistry of potential importance to an understanding of the biosynthetic origin, transport, storage, and release of these hormones. The characteristics of hormone binding to the neurophysins also have been used to make inferences about the interaction of these hormones with their cellular receptors.

Proposed Course of Action:
1) Continuation of studies on the quantitative aspects of neurophysin-hormone interaction, including the development and use of neurophysin-ligand affinity chromatographic systems and perhaps the pursuit of kinetic characterization.
2) Further investigation of the relationship between aggregation and neurophysin function.

Keyword Descriptors:

neurophysin; bovine neurophysins; equilibrium dialysis; sedimentation-equilibrium; conformational stability-neurophysins; neurohypophyseal peptide hormones.

Honors and Awards: None

Publications:

Chaiken, I.M., Randolph, R.E., and Taylor, H.C.: Conformational effects associated with the interaction of polypeptide ligands with neurophysins. Ann. N.Y. Acad. Sci. 248: 442, 1975.

Project No. Z01 AM 25,006-02 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Isolation, Purification and Chemical Characterization of the Human Neurophysins.

Previous Serial Number: NIAMDD-LCB-3

Principal Investigator: William M. McCormick

Other Investigator: Irwin M. Chaiken

Cooperating Units: National Pituitary Agency (Associated with University of Maryland School of Medicine and NIAMDD)

Man Years:

Total: .8
Professional: .1
Other: .7

Project Description:

Objectives:

1) To isolate and purify neurophysins from human posterior pituitary glands.

2) To study structure and hormone binding in order to: a) compare properties of human protein with more extensively studied proteins of other mammalian species, and b) correlate differences in properties with differences in structure, and thereby pursue an understanding of which structures are responsible for chemical properties.

Methods Employed: Isolation of neurophysins from human posterior pituitary tissue obtained from the National Pituitary Agency has proceeded according to methods previously established for other mammalian species. Final purification may include affinity chromatography, ion-exchange chromatography and preparative gel electrophoresis.

Binding of [^{14}C] labeled hormones and hormone analogues will be studied by equilibrium dialysis.

Binding kinetics may be studied by using stop-flow techniques.

Major Findings: The project was initiated in January and to date, findings are preliminary. Three major neurophysin components and several minor neurophysin components have been found in the crude neurophysin fraction. One additional major component of this fraction has been shown to have no affinity for a devised affinity chromatography system.

Significance to Biomedical Research: Study of the human neurophysins should be useful in determining their biological function. As more is learned of their peptide binding characteristics, a better understanding of protein-peptide interactions in general, should become apparent.

A better understanding of the mechanism of hypothalamic hormone systhesis, transport and controlled release may also be possible, all of which is important to understanding the functioning (on a molecular level) of the endocrine glands.

Proposed Course of Action:

1) Pursue the purification of the human neurophysins.
2) Study the possibilities of utilizing affinity chromatography both for purification and as a vehicle for studying binding interactions of matrix-bound hormones and hormone analogues with the neurophysins.
3) Study binding interactions by equilibrium dialysis.
4) Study binding kinetics.

Keyword Descriptors:

Human neurophysins, neurohypophyseal hormone binding proteins, affinity chromatography, equilibrium dialysis

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,007-02 LCB
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Proteinchemical and Immunological Studies of Bovine Neurophysin -(poly Ala-poly Lys)-Conjugates

Previous Serial Number: NIAMDD-LCB-3

Principal Investigator: Ernst A. Fischer

Other Investigators: John G. Curd
Irwin M. Chaiken

Man Years:

Total: 1.4
Professional: 1.4
Other: 0

Project Description:

1) To specifically couple purified bovine neurophysins I and II via protein amino groups to succinylated polyalanyl-polylysine.
2) To study the characteristics of binding of these conjugates to the vasopressin related ligand methionyl-tyrosyl-phenylalanine amide.
3) To study the immunological properties of these conjugates.
4) To raise antibodies directed against the neurophysins by injection of these conjugates in rabbits.
5) To purify and study the neurophysin antibodies.
6) To use these antibodies for conformational studies of the neurophysins and their ligand binding properties.
7) To isolate and study a possible neurophysin precursor with these antibodies.

Methods Employed: Coupling of the purified bovine neurophysins to the polymer polyalanyl-polylysine has been achieved by succinylation of the polymer, conversion of the carboxyl groups into hydrazide and subsequently into azide groups; these latter can react with the neurophysin amino groups.

The binding properties of these conjugates are studied using ^{14}C-labeled ligand and the method of equilibrium dialysis.

Rabbits were immunized with these conjugates.

Major Findings: Equilibrium dialysis experiments have shown that methionyl-tyrosyl-[^{14}C]phenylalanine-amide binds to the conjugates with about the same binding constant as that for the corresponding free neurophysin. The stoichiometry of the binding however seems to be 2:1 as compared to 1:1 in the free neurophysins; this distinction may be related to the molecular state of neurophysin in the bound versus free form.

Antibodies were elicited in rabbits against the neurophysin conjugates. In immunodiffusion assays these antibodies formed precipitin lines with the conjugates. They also react specifically with free neurophysin as judged from competition assays.

Significance to Biomedical Research: The methodology of specific protein-carrier immunogen preparation developed here may be useful in general, for studies of immunological properties of weak peptide- and protein antigens.

The studies undertaken should allow an increased understanding of the function of neurophysins as carrier proteins for neurohypophyseal hormones. They hopefully will cast light on the biosynthetic origin and fate of the neurohypophyseal hormones before entering the pituitary.

The protein-hormone complex could also serve as a model for hormone-receptor interaction.

Proposed Course of Action:

1) Continuance of binding studies using labeled oxytocin and vasopressin and conjugates of different degrees of substitution.
2) Isolation and characterization of anti-neurophysin antibodies.
3) Use of antibodies to isolate a possible neurophysin precursor.

Keyword Descriptors:

Neurophysin-(poly Ala-poly Lys)-conjugates, equilibrium dialysis, immunogenicity, immunodiffusion, rabbit antibody preparation.

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,008-12 LCB
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the relationship between the amino acid sequences and the three-dimensional structures of proteins: the mechanism of folding of protein chains and the mechanism of protein functions.

Previous Serial Number: NIAMDD-LCB-1

Principal Investigator: Hiroshi Taniuchi

Other Investigators: Diana S. Parker
Austine Davis
Carlo Di Bello

Man Years:
Total: 3.1
Professional: 1.0
Other: 2.1

Project Description:

Objectives: To find the mechanism of folding of protein chains on the basis of the amino acid sequence and to understand the dynamics of interatomic interactions underslying the function and regulation of proteins.

Working Hypothesis: Anfinsen and his colleagues have established that the amino acid sequence of proteins contains the information to determine the three-dimensional structure. On the basis of this information the mechanism of folding of disordered proteins into the ordered three-dimensional structure under physiological conditions is being intensively studied in many laboratories at home and abroad. The studies on the system of fragment complementation of staphylococcal nuclease and on the refolding of derivatives of bovine pancreatic ribonuclease A after reduction and reoxidation (see the previous reports) have led us into a model of protein chain folding as a working hypothesis. For example, the distribution of conformations of nuclease in 6 M guanidine HCl, pH 8 (intrinsic viscosity, 17 ml per g) may resemble a random coil. If the denaturant is removed, the distribution of conformations is assumed to change to another set of disordered conformations with equivalent energy. The intrinsic viscosity of this ensemble is assumed to be similar to that of a disordered fragment of residues 1 to 126, (Nuclease-(1-126) (10 ml per g) which is greater than that of native nuclease (2.4 ml per g). This hypothetical decrease in the domain of the polypeptide chain is assumed to be due to the increase in interactions between residues of short and long (distant in the amino acid sequence but near in space) ranges. However, the number of disordered states of the ensemble is assumed to be finite but still large in terms of the spatial orientation of the polypeptide chain backbone. The transition from the hypothetically disordered state to the native-like conformation (the activated state) in the ensemble would statistically occur. The energy of native conformation would then be lowered by the specific coupling of the local interactions between residues throughout the three-dimensional structure without a large change of conformation.

Methods Employed and Major Findings: The model predicts that the rate of refolding of disordered nuclease is determined by the probability of the folding of the disordered polypeptide chain to the native spatial arrangement and independent of the magnitude of stabilization energy of the native structure. Specific stabilization forces would be available only after completion of the folding. Experiments designed to test this hypothesis have been carried out since 1972, as described below. We have used the complementation system of the two overlapping fragments of nuclease, Nuclease-(1-126) and Nuclease-T-(50-149). As reported in the previous years, noncovalent interaction of Nuclease-(1-126) and Nuclease-T-(50-149) simultaneously yields, in 1 min, two alternative, enzymically active structures type I and II, resembling nuclease. The complementation is quantitative. In the type I structure Nuclease-T-(50-149) binds to the portion of Nuclease-(1-126) that contains residues 1 to 48. In the type II, Nuclease-(1-126) combines with the portion of Nuclease-T-(50-149) that contains residues 111 to 149. The redundant portions can be removed from the ordered portions of the complexes by digestion with trypsin in the presence of pdTp and Ca^{2+}. The derived complexes of type I and type II are composed of Nuclease-(1-48) and Nuclease-T-(50-149) and of Nuclease-(1-126) and Nuclease-(111-149), respectively. The population of type I and II complexes can be estimated by separating and quantitatively determining the component fragments of the two derived complexes. Type I complex is more stable against heat denaturation than the type II complex. The midpoints of the thermal transitions of type I and II complexes are approximately 33 and 23°, respectively. Therefore, it is expected that the population of type I complex is greater than that of type II at the thermodynamic equilibrium state of the system. On the other hand, if the folding of the two disordered polypeptide chains into the ordered complexes is statistical and independent of the stabilization force operating in the ordered structure, the ratio of population of the two complexes formed from the two fragments in 1 min may not necessarily be the same as that at the equilibrium state. In order to determine the initial ratio of type I to type II complex, we incubated an equimolar amount of Nuclease-(1-126) and Nuclease-T-(50-149) at pH 8 at 6° for 2 min. For the ratio at the equilibrium state, we incubated the mixture of the two fragments for 72 hours at 6°. After the incubation we added thymidine diphosphate and Ca^{2+} to the mixture. The mixture was digested with trypsin at 20 to 22° for 10 min. The digestion was stopped by the addition of Sepharose-soy bean trypsin inhibitor to the mixture. The mixture was filtered. Then, a small amount of free soy bean trypsin inhibitor was added to the filtrate in order to trap traces of trypsin which had escaped the affinity adsorption. The two derived complexes were separated from other material of the digestion mixture (the filtrate) by gel filtration on a Sephadex G-75 column at pH 8 at 6°. The two derived complexes thus obtained was completely free of trypsin contamination. The component fragments were then separated by gel filtration on a Sephadex G-50 or G-75 (see below) column, equilibrated and eluted with 30% acetic acid at 6°. The quantities of the fragments were determined by amino acid analysis and measurement of absorbance at 280 nm. The initial ratio of type I to type II complex is approximately equal (0.8 to 1) when the two complexes are formed by the interaction of the two fragments at 6°. However, the ratio of type I to type II complex changes to approximately 1.5 to 1 when the system reaches the equilibrium state at 6°. We have shown previously that the ligands, thymidine diphosphate and Ca^{2+} bind to the complexes and stabilize the structures against unfolding. The presence of ligands showed no effect on the initial ratio of the two complexes when they were formed from the two fragments. However, the ligands prevent the system from reaching the equilibrium state by suppressing the unfolding. After 72 hours incubation at 6° in the presence of the ligands, the ratio of type I to type II complex is still the same as the ratio at the initial complementation. The formation of the two complexes from the two disordered fragments at 24° is also approximately equal. The ratio of type I to type II complex changes to approximately 3 to 1 at the equilibrium state at 24°. We interpret the present results as follows. The thermodynamic equilibrium state of the system of the two complexes formed from Nuclease-(1-126) and Nuclease-T-(50-149) is attained through unfolding and refolding. The folding of the two disordered fragments into the spatial arrangement of ordered complexes is rather a statistical process and independent of the magnitude of stabilization energy of the ordered structures. In general the total potential energy of conformation of proteins is calculated as the sum of the energy contributed by individual interatomic interactions including hydrophobic interaction. That is, the interaction energy responsible for the formation of the native conformation is assumed to be additive. In contrast, we assume that the interatomic interactions specifically couple each other throughout the three-dimensional structure and reciprocally strengthen one another on thermodynamic principles of linked functions thus lowering the energy of the conformation. We also assume that such specific coupling of the interatomic interactions is operational only in the completed native three-dimensional structure under physiological conditions.

The system of complementing fragments of nuclease has afforded an excellent test object for studies of the relationship between the amino acid sequence and the three-dimensional structure as exemplified in the studies described above. These studies, including crystallization of the ordered complexes require a large amount of fragments in pure form. On the basis of the improvement of purification procedure of nuclease reported last year we have modified the methods (see the previous reports) of preparation of the fragments in order to obtain homogenous samples in high yields. The points of modification are as follows. Nuclease-T, composed of Nuclease-T-(6-48) and Nuclease-T-(49-149) or of Nuclease-T-(6-48) and Nuclease-T-(50-149), was obtained by limited digestion of nuclease with trypsin in the presence of ligands. The redundant segment (residues 99 to 110) of the complex (type II) formed from Nuclease-(1-126) and Nuclease-(99-149) was also removed by limited digestion with trypsin in the presence of ligands. The trypsin then was removed completely from the digestion mixture as a complex with soy bean trypsin inhibitor by gel filtration on a Sephadex G-75 column at pH 8. Nuclease-T was purified by affinity chromatography on a Sepharose-thymidine-diphosphate column of Cuatrecasas, Wilchek and Anfinsen, eluted with 0.5 M guanidine-HCl instead of ion exchange chromatography on a phosphocellulose column. The ordered complex of Nuclease-(1-126) and Nuclease-(111-149) was separated for the first time without partial dissociation by gel filtration on a Sephadex G-75 column at pH 8 at 6°. D.S. Parker has tested a Sephadex G-75 column with 30% acetic acid for separation of the fragments instead of a Sephadex G-50 column. She obtained a marked improvement in the resolution of Nuclease-(1-126) and Nuclease-T-(50-149) and in the separation of Nuclease-T-(50-149), Nuclease-T-(6-48) and Nuclease-(111-149) with a yield of approximately 80%. The method has been essential for the accurate quantitative determination of the ratio of type I and type II complexes described above.

For crystallization and X-ray diffraction studies the methods of preparation of reconstituted Nuclease-T (Nuclease-T') containing Nuclease-T-(6-48) and Nuclease-T-(49-149) have also been improved. Nuclease-T' was purified by affinity chromatography (see above), gel filtration successively on a Sephadex G-25 and a Sephadex G-75 column at pH 8 and ion exchange chromatography on a phosphocellulose column. The crystals formed from this preparation grew in a well defined form suitable for X-ray diffraction studies without degeneration in the crystallization solution (see the previous reports).

A precursor of nuclease containing an extra amino acid sequence of 18 residues presumably at the amino terminus of nuclease has been found. Nuclease V8 was purified from crude material (a phosphocellulose cake containing nuclease produced in the culture medium of the organisms) using ammonium sulfate fractionation, gel filtration on G-75 column and affinity chromatography. Nuclease V8 thus obtained showed the same specific activity with heat denatured DNA as a substrate as that of nuclease Foggi in pure form. However, ion exchange chromatography on a phosphocellulose column of the purified nuclease V8 yielded two fractions, A and B. Fraction A was eluted sooner and Fraction B later, both showing similar enzymic activities. The samples obtained from the two fractions were examined by amino acid analysis, measurement of sugar contents by the phenol-sulfuric acid method, polyacrylamide gel electrophoresis and two-dimensional peptide mapping after trypsin digestion. The results indicated that fraction B is the nuclease having the amino acid sequence determined previously. Fraction A contained two species. They are partially separable by ion exchange chromatography but are closely related to each other in terms of the amino acid sequence and appeared to contain extra residues attached to the NH_2-terminus of nuclease. No significant sugar content was found with either species. The extra residues were separated by gel filtration on a Sephadex G-75 column and paper electrophoresis after limited digestion of the mixture of fractions A and B with trypsin in the presence of the ligands. The extra amino acid sequences are composed of two tryptic peptides, (3 Asp, Thr, Ser, Glu, Gly, Val) Arg and (Asp, Thr, 2 Ser, Glu, Pro, Gly, Val) Tyr. It appears that the difference of the amino acid seq uence between fractions A and B is due to the presence and absence of amide group in the former tryptic peptide. The extra amino acid sequence of the precursor appears not to participate in the ordered structure of nuclease as is the case with residues 1 to 5 of nuclease. The presence of a similar precursor of nuclease Foggi also has been found.

A dimeric form of nuclease (Foggi) is formed under certain conditions. The specific enzymic activity of the dimer is approximately 8% of the native form. The dimer is separable from nuclease by gel filtration on a Sephadex G-75 column at pH 8. The dimer spontaneously converts, at least partially, to the monomeric form with full enzymic activity.

In the previous studies we observed that the structure of apo-cytochrome c is disordered. Apo-cytochrome c does not appear to bind noncovalently heme in a specific way to restore the ordered structure. Since the structure of heme is asymmetrical, the covalent bonds between the vinyl groups of the heme and cysteine residues 14 and 17 of apo-cytochrome c should be specifically oriented. Therefore, we predicted that an enzyme which recognizes the steric orientation of both heme and the segment of the polypeptide chain around residues 14 and 17 is probably involved in catalyzing the covalent attachment of the porphyrin ligand. B. Kandenbach (1970, Eur. J. Biochem. 12, 392-398) had suggested that apocytochrome c, after synthesis on the ribosomes, is transferred to the mitochondria and formation of the holoenzyme occurs in the mitochondria. E.M. Colleran and T.G. Jones (1973, Biochem. J. 134, 89-96) suggested that heme is directly attached to apocytochrome c. On the basis of this information, a search for an enzyme catalyzing the formation of covalent bond between heme and apocytochrome c has been initiated. Carlo Di Bello has synthesized Fe^{59}-labelled heme according to the methods of Colleran and Jones and prepared apocytochrome c by the method described in the previous report. Fe^{59}-labelled heme and apocytochrome c were incubated with the sonicated mitochondria preparation obtained from rat livers. Then, cytochrome c was added to the incubation mixture as a carrier. The mixture was fractionated by ion exchange chromatography (SP-Sephadex-C-25) and gel filtration (Sephadex G-50) (W. Fisher, H. Taniuchi and C.B. Anfinsen (1973) J. Biol. Chem. 248, 3188-3195). The incorporation of the radioactivity into the cytochrome c fraction was measured. The preliminary results indicated that a trace of cytochrome c may be synthesized from apocytochrome c and Fe^{59}-labelled heme in this system.

Significance to Biomedical Research: Our studies have revealed that the coupling of interatomic interactions in the three-dimensional structure of proteins provides the energy important for the stabilization of the structure. The studies also suggest that such coupling of the interatomic interactions is operational only in the completed native structure. This concept bears a similarity to that known as the so-called allosteric phenomenon. In allosteric interaction an interaction system between the residues and a ligand at one site of a protein molecule reciprocally interacts with the interaction system between the residues and another ligand at another site via the intractions between the subunits. This similarity suggests that the folding of protein chains (the decrease in the energy of the conformation) and the allosteric phenomenon (the linkage of the two interatomic interaction systems at a distance apart in the three-dimensional structure) may have the same origin, that is, the coupling of the interatomic interactions throughout the three-dimensional structure. The studies of the dynamics involved in this specific coupling of the interatomic interactions is important for understanding the structure-function relationship, the regulation of function of biological macromolecules and the interaction between the macromolecules. The studies will aid in the prediction of the functional three-dimensional structure of a protein on the basis of the amino acid sequence.

Proposed Course of Project:

1) Determination of the atomic coordinates of type I complex (Nuclease-T') by X-ray diffraction study.

2) Crystallization and determination of the atomic coordinates of type II complex by X-ray diffraction study.

3) Studies on the interatomic interactions on the basis of the atomic coordinates of type I and type II complexes and the kinetic and thermodynamic parameters involved in the conformations.

4) Studies on the mechanism of the enzymic activity of nuclease of the basis of the atomic coordinates of type I and type II complexes.

5) Search for an enzyme involved in the formation of cytochrome c from apocytochrome c and heme.

Keyword Descriptors:

Staphylococcal nuclease, precursor of nuclease, fragments of nuclease, complementation of nuclease fragments, ordered complex of fragments, simultaneous formation of two complexes from the two overlapping fragments, Nuclease-T, type I complex, type II complex, crystals of Nuclease-T', preparation methods of fragments, X-ray crystallography of complexes, mechanism of folding of protein chains, coupling of interatomic interactions, stabilization energy, statistical folding of protein chains, origin of stabilization energy, origin of allosteric phenomenon, cytochrome c synthesis, F^{59}-labelled heme, apocytochrome c, mitochondria.

Honors and Awards: None

Publications:

Taniuchi, H. and Bohnert, J.L.: The mechanism of stabilization of the structure of Nuclease-T' by binding of ligands. J. Biol. Chem. 250: 2388-2394, 1975.

Bohnert, J.L. and Taniuchi, H.: The purification of staphylococcal nuclease by an improved method. J. Biol. Chem. 250: 2394, 1975.

Project No. Z01 AM 25,009-01 LCB
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the relationship between the amino acid sequences and the three-dimensional structure of proteins: the methanism of formation of "correct" disulfide bonds in proteins after reduction and reoxidation.

Previous Serial Number: None

Principal Investigators: Seetharama A. Acharya
Hiroshi Taniuchi

Man Years:
Total: 1.1
Professional: 1.1

Project Description:

Objectives: To elucidate the mechanism of folding of proteins on the basis of amino acid sequence, with special reference to the formation of correct disulfide bands.

Methods Employed and Major Findings: The two well recognized states in the conformation of a protein are the native and the disordered form. The disulfide cleaved polypeptide is apparently a disordered form and has been shown to differ from the native molecule in many of its hydrodynamic properties. For example, we observed previously that the randomly oxidized Ribonuclease-A is less retarded than the native species on gel filtration (see the previous report, NIAMDD-LCB-1, 1971-1972). A study has been made to see whether this difference in the hydrodynamic properties of a disordered polypeptide chain and its native form is generally sufficient enough to fractionate the native molecules from the nonnative molecules by molecular sieve chromatography, simply based on their hydrodynamic volumes. The fully reduced lysozyme eluted from the Biogel P-30 columns at a position intermediate to that of trypsinogen, and the native lysozyme, suggesting that molecular sieve chromatography separates the native and the nonnative species, based on their hydrodynamic volumes. Randomisation of the "correct" disulfide pairing of the native lysozyme using catalytic amounts of β-mercaptoechanol under denaturing conditions has been observed using this chromatographic procedure.

Air oxidation of reduced hen egg white lysozyme in absence of β-mercaptoethanol, regenerated only 60 to 65% of the enzymatic activity. This fully air oxidized sample of lysozyme, showed two populations of hydrodynamic volume; one of these appeared to be the native form, eluting at the position corresponding to that of the native lysozyme; the other form eluted at the position corresponding to that of the reduced lysozyme and was enzymatically inactive. Both these fractions had no detectable amounts of free sulfhydryl groups. Acceleration of the rates of oxidation of the -SH groups of reduced protein using catalytic amounts of Cu^{++} decreases the yield of native form with a concomitant increase in the inactive forms. Thus when the rate of oxidation is fast, the reduced polypeptide chain is converted to fully oxidized, disordered polypeptide chain, without any significant changes in the hydrodynamic volume. The inactive form can be rearranged to the native form using β-mercaptoethanol or with an equimolar amount of reduced lysozyme. These results clearly suggest that the disulfide interchange is essential for the correct disulfide pairing in reduced lysozyme upon oxidation as was the case with reduced RNase (the report, NIAMDD-LCB-1, 1971-1972).

The rate of formation of lower hydrodynamic structure appeared to be very high, during the intial stages of air oxidation, and this rate falls off very rapidly as the concentration of the free sulhydryl groups in the reaction mixture decreases. The lower hydrodynamic volume species formed at the early stages of Cu^{++} catalyzed air oxidation of reduced lysozyme had some free sulfhydryl groups in them; which could be trapped with iodoacetic acid or iodoacetamide. The lower hydrodynamic volume species labelled at these free sulfhydryl groups with radioactive iodoacetic acid have been prepared. The radiolabelled species were purified by ion exchange chromatography on Biorex-70 columns. These species had nearly 40 to 50% of the specific activity of the native proteins. In order to identify the -SH groups alkylated with iodoacetic in these species, these proteins were reduced, carboxymethylated with cold iodoacetic, digested with trypsin and the tryptic peptides were analyzed on Dowex columns. Characterization of the radioactive tryptic peptides by electrophoesis, paper chromatography and amino acid analysis enabled the identification of the cysteine residues which were free in the lower hydrodynamic volume species. Chemical analysis of the tryptic peptides of these species showed that at least two types of molecular species with one open disulfide bond formed during the reoxidation of the reduced lysozyme have a compact lower hydrodynamic volume structure. The major molecular species is the one in which the cysteine residues at position 6, and 127 are not paired. In the other derivatives, one or more of the cysteine residues at positions 76, 80 and 94 are not paired. Apparently even when these disulfide bonds are not formed, the molecules with the other three disulfide bonds are capable of forming the stable, compact native-like structures of the protein.

Significance to Biomedical Research: The three-dimensional structures of the native protein is apparently formed and stabilized by the noncovalent interactions of the polypeptide chain. The previous reports and the other report of this year as well as the present study indicate that the interatomic interactions responsible for the formation of the native structure operate cooperatively throughout the three-dimensional structure. Individual interactions are, however, not equivalent in their contribution towards the stabilization of the native conformation of the protein. An investigation of the mechanism of formation of "correct" disulfide bonds in proteins is of help in understanding the mechanism of folding of the disordered polypeptide chain into the native structure. The information obtained from these studies will contribute to a more precise definition of the nature of the interatomic interactions maintaining the integrity of the three-dimensional structure of the protein, and will also aid in the prediction of the three-dimensional structures of a protein on the basis of amino acid sequence.

Proposed Course of Research:

1) Characterization of the nature of the disulfide bonds in the higher hydrodynamic volume forms.

2) Reduction and reoxidation of the derivative of lysozyme containing carboxymethylated cystine residues at position 6 and 127.

3) Crystallization and X-ray diffraction studies of the above derivatives.

4) Carboxypeptidase and aminopeptidase digestion of the derivative carboxymethylated at cysteine residues 6 and 127 in an attempt to get a complementing system to explore further the folding process of reduced lysozyme.

Keyword Descriptors:

lysozyme, Cu^{++} catalyzed air oxidation of -SH groups, disulfide pairing and interchange, peptide mapping, hydrodynamic volume, molecular sieve chromatography.

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,010-02 LCB
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the relationship between the primary and tertiary structure of protein: the crystallization and X-ray diffraction study of semisynthetic nuclease T'.

Previous Serial Number: NIAMDD-LCB-2

Principal Investigators: Robert A. Vigna
Hiroshi Taniuchi

Cooperating Unit: Christian B. Anfinsen
(NIAMDD, LCB, Protein Chemistry)

Man Years:

Total: 1.2
Professional: 1.1
Other: 0.1

Project Description:

Objective: To crystallize and define by X-ray diffraction techniques the three dimensional structure of semisynthetic nuclease T'.

Rationale: The rationale underlining this work is presented in detail in the report of the previous year. The key points are summarized here.

Nuclease T, an enzymically active complementing system of staphylococcal nuclease derived by digestion of liganded nuclease with trypsin, can be reconstituted from its component peptides (nuclease-T-(6-48) and nuclease-T-(49, 50-149). Reconstituted nuclease T(nuclease T') is identical to nuclease T by several criteria.

Nuclease-T-(6-47) has been synthesized by Merrifield solid phase methods. Semisynthetic nuclease T' (synthetic nuclease-T-(6-47/48) + nuclease-T-(49, 50-149) has been obtained in a highly purified state.

Crystals of nuclease T' suitable for X-ray diffraction analysis have been prepared and shown to be isomorphous with those of nuclease. A complete X-ray crystallographic structure of nuclease T' will be undertaken in this section. Tertiary structural information obtained will be correlated with the results of fragment exchange experiments performed on the nuclease T' system (see previous reports for details).

The availability of semisynthetic nuclease T' crystals will demonstrate the possibility of obtaining a largely synthetic enzyme in chemically pure form using solid phase synthetic methodology. The identity of semisynthetic nuclease T' crystals to those of nuclease T' will be supported by X-ray diffraction analysis. The proposed X-ray analysis of nuclease T' in conjunction with the thermodynamic information derived from nuclease T' fragment exchange experiments suggest the immediate extension of these studies to crystalline semisynthetic nuclease T'.

Methods Employed and Major Findings: Throughout the course of this work, nuclease, nuclease T, nuclease-T-(6-48), nuclease T-(49-149) and nuclease-T-(50-149) have been prepared as required. Nuclease-T-(49,50-149) is now routinely passed over pdTp-sepharose to remove nuclease-(6-149) known to contaminate this preparation. A detailed description of the preparative methods employed are included in the report of the previous year.

The synthesis of nuclease-T-(6-48) was performed using the Merrifield solid phase technique with a protocol altered substantially from that published previously for this fragment. Modifications reported by various authors designed to improve the overall yield of target peptide were incorporated where practicable into the synthetic scheme. Changes involved a reduced degree of esterification of the protected COOH terminal residue (lysine 48) to the solid support, use of 25% TFA containing indole and use of methylene chloride as the solvent for triethylamine. In addition, an extensive washing procedure was inserted between the two coupling periods for each t-Boc amino acid to be incorporated and an improved protecting group for the phenolic side chain of tyrosine, (O-2,6-dichlorobenzyl ether) was also used. However the yield of synthetic nuclease-T-(6-48) was not improved as anticipated using this revised scheme.

The peptidyl resin was cleaved with HF, deblocked and chromatographed on G-25F (Sephadex) as previously published. The major fraction recovered from gel filtration contained synthetic peptide capable of generating 0.6% the activity of nuclease T' against heat denatured DNA when complemented with nuclease-T-(49,50-149). The synthetic nuclease-T-(6-48) recovered from G-25F was applied to a (3cm X 300cm) column of G50F (Sephadex) and eluted with 0.05M ammonium acetate, pH 8.0. The synthetic peptide which chromatographed in the same elution volume as nuclease-T-(6-48) was 2% as potent as nuclease-T-(6-48) in yielding an active complex with nuclease-T-(49,50-149). Amino acid analysis of G-25F and G50F recovered synthetic nuclease-T-(6-48) gave a composition which compared favorably in most cases to that of nuclease-T-(6-48) and with the analysis for synthetic nuclease-T-(6-47) published previously. Two dimensional peptide mapping of these partially purified preparations after digestion with trypsin indicated the presence of nearly all the characteristic tryptic peptides of nuclease-T-(6-48) in addition to a number of other fragments arising from the 'failure sequences' expected to occur during the synthesis. One major tryptic fragment covering the sequence of nuclease-T-(6-48) from residues 36 to 45 was not observable at this level of purification. The synthetic nuclease-T-(6-48) recovered at this stage was capable of forming an enzymically active semisynthetic complex with nuclease-T-(49,50-149) which, in the presence of the ligands pdTp and calcium, was shown to be resistant to digestion by trypsin.

Synthetic nuclease-T-(6-48) fractions obtained after G-25F or G-50F chromatography which yielded active semisynthetic nuclease T' complex with pdTp-Sepharose treated nuclease-T-(49,50-149) were pooled. These fractions (400 mg) represented 20% of the total crude material recovered. Half of this material was incubated with 20 mg of nuclease-T-(49,50-149) at room temperature overnight in 0.1 M NH_4Ac (5 mM $CaCl_2$) pH 8.0 and applied to a pdTp-Sepharose column at 6°C. The specific activity of the semisynthetic nuclease T' (henceforth S-Nase T') recovered after elution with 0.5 M guanidine HCl and desalted on Sephadex G25F was 20.7 ΔA_{260}/min/mg. This preparation was applied to a G50F column eluted with 30% HAc to recover the synthetic nuclease-T-(6-48) bound in complex. Polyacrylamide gel electrophoresis of S-NaseT' obtained by affinity methods indicated the presence of peptide material that bound nonspecifically to pdTp-Sepharose and was eluted with guanidine HCl. The profile obtained after G50F chromatography also reflected this observation. The amino acid analysis and two dimensional tryptic peptide map of the synthetic nuclease-T-(6-48) isolated in this manner showed marked improvement over that obtained for the crude synthetic preparation. The tryptic fragment of nuclease-T-(6-48) covering the residues 36 thru 45 in the sequence was clearly evident in the peptide map made at this stage of the purification.

The remaining half of the synthetic nuclease-T-(6-48) was processed similarly with the exception that the complex was passed twice through pdTp-Sepharose, desalting after each passage. The specific activity of the 2.1 mg of S-NaseT' recovered by this procedure was 39 ΔA_{260}/min/mg. Synthetic nuclease-T-(6-48) (0.26 mg) recovered after G50F chromatography will not permit further purification which at this point was to have been digestion of the complex resulting after complementation with nuclease-T-(50-149) in the presence of the ligands pdTp and calcium by trypsin.

Synthetic nuclease-T-(6-48) is again being prepared at this time following more closely the schedule previously published for this synthesis. The progress of this synthesis is being monitored by ninhydrin assay and periodic amino acid analysis of the peptide resin. In conjunction with this synthesis a study of the synthetic peptide 36 thru 48 is being undertaken to determine what improvements if any can be made in the synthesis of this region which will benefit the overall yield of the complete synthetic nuclease-T-(6-48) peptide.

The synthetic nuclease-T-(6-48) prepared above will be purified as outlined. This synthesis is designed to yield approximately four times the crude peptide material prepared previously. A systematic purification and characterization scheme will be followed. Amino acid analysis, peptide mapping and enzymic activity will be the principal methods used to check the progress of the purification. Synthetic nuclease-T-(6-48) recovered after the final functional purification step if chemically identical to nuclease-T-(6-48) will be used for the crystallization of S-NaseT' complex consisting of synthetic nuclease-(6-48) and nuclease-T-(49-149).

Significance to Biomedical Research: Observations described in previous reports from this section suggest that the interatomic interactions responsible for the formation of the native structure of a protein operate cooperatively throughout the three-dimensional structure. The X-ray crystallographic investigation of SNaseT' and selected analogues supplemented with the thermodynamic information obtainable through labelled fragment exchange experiments should prove to be an effective combination of methods for studying the interatomic interactions underlying the structure of proteins. These studies will contribute to a more precise definition of the nature of interatomic interactions which are essential for maintaining the structural and functional integrity of proteins.

Improvements in the synthetic method made during the course of this work may contribute substantially to the technology designed to produce clinically useful synthetic hormones and enzymes.

Proposed Course of Research:

1) Crystallization and X-ray diffraction study of semisynthetic nuclease T'.

2) Crystallization, X-ray diffraction and thermodynamic studies on semisynthetic nuclease T' analogues composed of selectively modified synthetic nuclease-T-(6-48) with nuclease-T-(49-149).

3) Systematic study of synthetic methods intended to improve the overall yield of peptides required for the proposed studies on the semisynthetic nuclease T' system.

4) Total synthesis, crystallization and X-ray diffraction study of nuclease T'.

Keyword Descriptors:

solid phase peptide synthesis, X-ray crystallography, staphylococcal nuclease, nuclease T, semisynthesis of proteins, affinity chromatography, gel filtration chromatography, protein folding, peptide mapping, functional purification of proteins.

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,011-01 LCB
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein unfolding by fragment exchange with a system derived from cytochrome c.

Previous Serial Number: none

Principal Investigator: Roy R. Hantgan
Hiroshi Taniuchi

Person Years:

Total: 0.8
Professional: 0.8

Project Description:

Objective: Estimation of the stabilization energy maintaining the structure of cytochrome c; finding a relationship between the stabilization energy and atomic co-ordinates of the protein.

Methods Employed and Major Findings: Corradin and Harbury reported that the two fragments of cytochrome c, residues (1-65) containing the heme and residues (66-104), interact to form an ordered complex similar to the native structure (Corradin, G., and Harbury, H.A. (1971) PNAS 68, 3036).

Preliminary experiments in this laboratory have shown that a heme peptide of residues (1-65) derived from horse heart cytochrome c by cyanogen bromide cleavage can interact non-covalently with the heme free apoprotein of cytochrome c to form a complex with spectral properties similar to those of the native protein (see NIAMDD-LCB-3, 1971-1972). The purpose of this project is to use the method of fragment exchange with this complementing system to determine rates of unfolding by studying the reaction:

$(1\text{-}65)^*(1\text{-}104) + (1\text{-}65) \rightleftharpoons (1\text{-}65)(1\text{-}104) + (1\text{-}65)^*$ (in which * denotes a radioactive label). As unfolding of the native-like structure of the complex will be rate limiting in the above reaction, the rate of fragment exchange will provide a direct measurement of the unfolding process.

In order to accomplish this, it is necessary to purify native cytochrome c, and prepare the heme fragment (1-65) and the heme free apoderivative of cytochrome c. Then, further experiments to define the nature of the interactions responsible for the complementing system can be done. Finally, an immunoabsorbent system must be devised which can quantitatively remove one component from the exchange reaction so that the extent of incorporation of the label into the complex can be determined.

A. Preparation of the components of the complex

Native cytochrome c is purchased from Sigma Chemical Co. Commercial samples contain autoxidizable deaminated and polymeric forms of the native protein, which are removed by a combination of gel filtration and cation exchange chromatography to yield a native, monomeric protein.

Silver sulfate is used to remove the heme moiety from cytochrome c; the resultant silver mercaptide bonds are cleaved with a thiol reagent, the free sulfhydryl groups blocked with an alkylating reagent, and the product purified by gel filtration chromatography.

Cyanogen bromide cleavage is used to produce the heme fragment of residues (1-65). Gel filtration in 30% acetic acid is used to separate and purify the desired peptide.

B. Characterization of the complex

The visible absorbance spectrum of cytochrome c in the neighborhood of 400nm, the Soret region, is a sensitive indicator of the liganding of the heme and the overall conformation of the protein. For example, λ max for native cytochrome c is 409-10 nm in the oxidized (ferri) form, whereas denatured forms exhibit λ max at 407nm. Likewise heme fragment (1-65) shows a 407nm peak, the magnitude of which decreases upon titration with an equimolar quantity of the apoprotein, indicating some form of complex formation.

The heme fragment (1-65) is readily autoxidizable in the reduced (ferro) state, with λ max at 417nm in contrast to the stable ferro form of native cytochrome c with λ max at 415nm. But reduction of the aforementioned ferri complex (1-65)(1-104) shifts its spectrum to coincide with that of native cytochrome c. This coincidence persists when the complex (1-65)(1-104) is reoxidized, as the spectrum now shows λ max at 410, typical of a native-like conformation.

Thus a spectral investigation indicates the complementing system can display properties of the native protein. The resistance of ferro cytochrome c to tryptic digestion has been used in experiments to remove possible redundant portions of the (1-65)(1-104) sequence. By spectral criteria, coupled with peptide mapping, it has been shown that a brief tryptic digestion removes some of the redundant portions of the complex without altering the basic structure of the molecule.

With this assurance, a preparative scale experiment was carried out in an effort to isolate the trypsin surviving complementing system by gel filtration chromatography. The separation at present does not permit a unique identification of the cleavage points, though this appears technically possible.

C. Immunoabsorbent system

As one approach to the separation problem, a high molecular weight (>100,000) glutaraldehyde cross linked derivative of cytochrome c is being used to elicit antibodies (in rabbits) specific for native cytochrome c. As the antisera does not appear to cross reactive with the heme fragment (1-65), this shows promise for the proposed immunoabsorbent analytical system.

Also, the disordered apo cytochrome c has been used to produce rabbit antisera, although the specificity of these antibodies may not be narrow enough to distinguish the components of the complex.

At present production and collection of antisera for future fractionation is being continued.

Significance to Biomedical Research: The mechanism of protein biosynthesis, the relationship between protein structure and function, and the correspondence between the amino acid sequence of a protein and its unique tertiary structure are problems which can be studied by the technique of fragment exchange. The method is unique in that it permits a measure of unfolding rates under physiological as well as denaturing conditions. Previous pertubation methods require substantial disruption of native structure in order to determine unfolding rates.

Finally cytochrome c is of interest for the evolutionary invariance of its amino acid sequence, its position in the election transport chain, and its well studied structure. Sufficient information is currently available so that this study may provide the answers to many interesting questions of protein conformation and the underlying interatomic interactions.

Proposed course of research:

1) Determination of which portions of the complementing system are necessary for its structure, by trypsin digestion, fractionation and isolation of the components.

2) Development of an immunoabsorbent system by fractionation of the rabbit antisera.

3) Study exchange rates as a function of temperature, pH, denaturant concentration, and modifications in the amino acid sequence of the complementing system.

4) Crystallization and X-ray diffraction studies of the complex.

Keyword Descriptors:

cytochrome c, cyanogen bromide cleavage, trypsin digestion/peptide mapping, complementing system, protein folding, antibody specificity, cytochrome c as an antigen, difference spectroscopy, apo cytochrome c, fragment exchange.

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,012-05 LCB
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nuclear magnetic resonance studies of the structure and function of staphylococcal nuclease

Previous Serial Number: NIAMDD-LCB-12

Principal Investigator: Alan N. Schechter

Cooperating Units: Division of Computer Research and Technology (Richard Feldmann)

Man Years
Total: 0.1
Professional: 0.1

Project Description:

Objectives: To use NMR methods to study the geometry of the active site of staphylococcal nuclease in solution so as to understand better its mechanism of action.

Methods Employed: As outlined in the last annual report continuous wave and FT NMR spectra have been obtained of nuclease. Paramagnetic ions (such as Gd(III)) have been used as perturbants of the NMR resonances. This approach has allowed the accurate measurement of distances within the protein and between the protein and a substrate analog, pdTp. These distances have been used, with a computer modeling program, to generate a solution geometry for nuclease and its substrate analog.

Major Results: The geometry established for the protein in solution is very similar to that in the crystal structure, with the exception of the location of the methyl group on the substrate analog. The reasons for this discrepancy are not clear.

Data for several other substrate analogs and several substrates have been collected for similar analyses.

Significance to Biomedical Research: High resolution NMR can be used to understand details of the structure and function of proteins at a level comparable to that of crystallography and, in addition, can be used to obtain kinetic (or dynamic) information. Full understanding of enzyme mechanisms and protein structure in solution requires such approaches.

Proposed Course of Project: The data collected will be prepared for further publications. Beyond that the project has been temporarily discontinued.

Keyword Descriptors:

Staphylococcal nuclease, active site, paramagnetic ions

Honors and Awards: None

Publications:

Griffin, J.H., Furie, B., and Schechter, A.N.: Application of nuclear magnetic resonance spectroscopy to proteins. Biochimie, in press.

Project No. Z01 AM 25,013-04 LCB
1. Lab. Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunological Properties of Staphylococcal Nuclease.

Previous Serial Number: NIAMDD-LCB-13 and 14

Principal Investigators: John Curd
John Gerlt
Alan N. Schechter
Christian B. Anfinsen

Man Years:

Total: 1.4
Professional: 1.4

Project Description:

Objective: Previous investigations in this laboratory have fractionated goat antibodies, produced against staphylococcal nuclease, by using available proteolytic fragments of nuclease. After fractionation two nonprecipitating populations were obtained which were operationally specific for antigenic determinants in the amino acid sequences 99-126 and 127-149. The antibodies to region 99-126 inactivate nuclease and the k_{on} and K_{assoc} of this inactivation have been determined. A model for conformational equilibria of polypeptides in solution has been proposed and empirical constants for these equilibria have been determined.

The current work attempts to extend our understanding of the nature of the interaction of antibodies with nuclease and to utilize solid phase peptide synthesis and affinity chromatography to isolate antibodies specific for other amino acid regions of nuclease.

The interaction of anti-(99-126) with the enzymatically active complex nuclease T' (complexing fragments 6-48 and 50-149) is being studied in an attempt to measure the effect of anti-(99-126) on the dissociation rate of 6-48 from 50-149. The determination of dissociation rates in the presence and absence of anti-(99-126) should show if anti-(99-126) stabilizes the "folded" or native conformation of 50-149 and slows dissociation from 6-48 as predicted from thermodynamic considerations.

Methods and Major Results: Antibodies to native nuclease have been prepared in goats as previously described. These antibodies have been fractionated by sequential immunoadsorption on Sepharose columns to which nuclease and several of its polypeptide fragments have been covalently attached. Several polypeptides from the sequence of nuclease including 53-69, which is a helix is a helix in the native protein, have been produced by the Corley modifica- of the Merrifield procedure.

The nuclease fragments 6-48 and 50-149 have been prepared and carbamylated with ^{14}C-cyanate. The carbamylated fragments have been recomplexed to form a radiolabeled, enzymatically active complex identical to nuclease T'. This material has been isolated from inactive material by affinity absorption on pdpT-sepharose. The dissociation rate of the complexed radiolabeled nuclease T' has been determined and is consistent with previous results obtained by a different method. Antibodies specific for region 99-126 slow the dissociation of the radiolabeled complex. However the preparation of 99-126 does not seem to be operationally "mono-specific" when analysed by gel chromatography.

A second antibody population specific for the α-helical region 53-69 has been isolated by affinity adsorption on a column of Sepharose coupled with synthetic peptide corresponding to the sequence 53-69. Operationally mono-specific antibodies have been eluted from this column by 1 mM solutions of the peptide in buffered saline. Elution with acid or denaturing reagents did not yield completely mono-specific antibody populations. The anti-(53-69) was separated from excess peptide by gel filtration. The anti-(53-69) inactivates nuclease and consists of at least 2 populations of antibodies with association constants of $\sim 10^{10}$ M^{-1} and $\sim 10^{8}$ M^{-1}.

Significance to Biomedical Research: The study of the interaction of anti-(99-126) extends our understanding of antibody interaction with antigenic determinants on polypeptides in solution. It will test thermodynamically whether anti-(99-126) is or is not recognizing a "folded" or native determinant.

The demonstration that solid phase peptide synthesis and affinity chromatography can be employed to isolate antibodies specific to regions of a polypeptide of known sequence is important because it extends the range of immunochemical studies of proteins of known sequence. One is no longer limited by the availability of proteolytic or chemical cleavage peptide fragments. Theoretically the investigator can now pick the sequence or structure to be studied and isolate antibody populations specific for that structure.

Proposed Course of the Project: The study of the interaction of anti-(99-126) will be extended to the measurement of the association and dissociation rates of monospecific anti-(99-126) with nuclease T'. The affinity chromatography - solid phase peptide synthesis method of isolation of antibodies is being incorporated into our study of the immunology of hemoglobins.

Keyword Descriptors:

Immunochemistry, antibodies, solid phase peptide synthesis, staphylococcal nuclease, affinity chromatography, nuclease, peptide complementation

Honors and Awards: None

Publications:

Sachs, D.H., Schechter, A.N., Eastlake, A. and Anfinsen, C.B.: An immunological distinction between the possible origins of enzymatic activity in a polypeptide fragment of staphylococcal nuclease. Nature 251: 242-244, 1974.

Furie, B., Schechter, A.N., Sachs, D.S., and Anfinsen, C.B.: An immunological approach to the conformational equilibria of staphylococcal nuclease. J. Mol. Biol. 92: 497-506, 1975.

Project No. Z01 AM 25,014-01 LCB
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Functional Properties of the Immune Response Gene to Nuclease.

Previous Serial Number: none

Principal Investigators: Jay A. Berzofsky
Alan N. Schechter

Cooperating Units: Immunology Branch, NCI (David H. Sachs and Gene M. Shearer)
Lab. of Chemical Physics, NIAMDD (Hideo Kon)

Man Years:

Total: 1.1
Professional: 1.1
Others: 0

Project Description:

Objectives: It has previously been shown that immune response genes (Ir genes) which map within the major histocompatibility complex (H-2) of mice determine the ability of various inbred strains of mice to respond to diverse antigens with antibody production and/or cellular immunity. These are apparently a separate level of regulator genes, which have the ability to recognize and distinguish a broad range of immunogenic determinants. In synthetic polypeptide antigen studies these determinants have generally been identified with backbone or "carrier" determinants. However, natural protein antigens have not been widely studied, and it is not clear what part of the molecule might serve this "carrier" function, or how it might be recognized. One objective of this study is to understand these mechanisms.

It has previously been shown by some of the present investigators that the immune response to the natural protein antigen, staphylococcal nuclease (henceforth "Nase"), whose structure has been well studied, is under H-2-linked Ir-gene control. This response is being investigated from two different points of view: 1) By studying the relative proportions of antibodies to different antigenic determinants on Nase made under Ir-gene control by high responder and low responder strains, and 2) by studying the immune response in appropriately chosen strains to polypeptide fragments of Nase each bearing only one or a few of the several antigenic determinants on the whole Nase molecule.

Major Results and Methods Employed: To obtain a preparation pure enough to use as an immunogen, several modifications of the preparative procedure for

Nase were studied. The resulting preparation was pure by polyacrylamide gel and SDS-polyacrylamide gel electrophoresis and by Ouchterlony immunodiffusion in agar using antisera to less purified Nase, and by specific enzymic activity.

For the first aspect of the project, two high responder strains of mice (A/J and SJL) were immunized and boosted five times with this highly purified nuclease. The resulting sera were analyzed for 1) antibodies to native Nase by an assay using inhibition of enzymic activity of Nase and 2) antibodies to a polypeptide fragment of Nase (the "E" fragment) consisting of the C-terminal third of the molecule (residues 99-149). In order to do the latter, this "E" fragment was prepared and purified and characterized by two-dimensional peptide mapping and by enzymic activity when complexed with the complementary N-terminal portion (residues 1-126) of Nase. Some of this "E" fragment was radioactively labeled by specific carbamylation of the N-terminal -amino group with ^{14}C-KCNO. A radioimmunoassay was then developed for antibodies to this "E" peptide by a micro-scale modification of the polyethylene glycol method of Desbuquois and Aurbach. Early results have shown that both of the high responder strains studied (A/J and SJL) produce about the same fraction (about 10%) of antiNase antibodies which are directed against the E region.

For the second aspect of the study, three groups each of five different strains of mice (3 high and 2 low responder types) have been immunized as follows: one group primed and boosted several times with the purified E fragment; one group primed with native Nase and then boosted several times with the purified E fragment; and one group primed with native Nase, not boosted at all, but bled as a control at the same time that the second group of mice are bled after each boost. The sera from individual mice of all 3 groups of each of the five strains (total of 100 mice) are collected separately after each immunization and are to be assayed individually (in order to be able to detect statistically significant differences between groups) for both antibodies to whole Nase and antibodies to E fragment.

In the course of this work, the effect of polyethylene glycol on the folding of the "E" fragment was studied by circular dichroism. No increase in helix content over the random peptide in water could be detected.

Another sub-project of the study was to answer the question of whether proteins emulsified in complete Freund's adjuvant (consisting of mineral oil, an emulsifying agent, and killed tubercle bacilli) remained "native" at the time of immunization. Various optical spectral probes (including absorption and fluorescence and circular dichroism) of proteins are impossible to perform on a water-in-oil emulsion because of infinite scattering. However, hemoglobin, which is far more labile than nuclease and therefore a better test of denaturation in complete Freund's adjuvant (CFA), also has the advantage of the paramagnetic heme which can be studied by electron paramagnetic resonance (EPR). Therefore we undertook to study the EPR of both oxy and high spin ferric hemoglobin after emulsification and incubation in CFA for 2 hrs. or 24 hrs. at 4°C. We have found that even after 24 hrs. in CFA, neither oxy nor ferric hemoglobin shows denaturation, and in fact the oxy is so little perturbed by the CFA that no increase in oxidation to the ferric form can be detected over that of oxyhemoglobin incubated in aqueous buffers for the same times.

Significance to Biomedical Research: First, from a basic point of view, Ir gene regulation is another whole level of recognition which has great breadth and specificity just as immunoglobulins do, and yet appears to have a separate and perhaps different set of recognition sites from those of immunoglobulins for the same chemical antigens.

Secondly, from the clinical point of view, the immune response genes are of importance in understanding and potentially modifying man's ability to eliminate clones of his own cells which have become malignant, and his not uncommon failure to prevent an immune response to his own cells and tissues in the many diseases of autoimmune origin.

Finally, the demonstration that a fairly labile protein such as hemoglobin is not detectably denatured by the emulsification process (e.g. surface denaturation) or the components of complete Freund's adjuvant itself is of value to biochemists and immunologists who would like to use immunochemical techniques to study protein structure and conformation.

Proposed Course of Project:

1) The antisera to native Nase produced by the 2 high responder strains are to be further characterized by fractionation on affinity columns of various fragments of Nase as well as by techniques to measure affinity constants. The small amount of antibodies produced by low responder strains are also to be studied by similar approaches to see if they produce smaller quantities of the same spectrum of antibodies in the same proportion as high responder strains, or whether they are "low" responders to whole Nase by failure to produce antibodies to some major determinants to Nase (i.e., nonresponders to these) even though they produce the same amounts of antibodies to other determinants as do the high responders. If the latter were true, it would explain why, when assayed against whole Nase with many determinants, they appear to be "low" responders rather than nonresponders.

2) The individual sera of animals immunized with E fragment or primed with whole Nase and boosted with E fragment are to be further characterized.

3) Both types of approaches are to be extended with other polypeptide fragments representing other regions of Nase, pending the results of the studies with the E fragment.

4) The same phenomena in cellular immunity are to be studied by the footpad assay of S. Davis and co-workers.

5) The implications of some findings during the course of development of the radioimmunoassay regarding the kinetics of the antigen-antibody association reaction and its significance for the conformational equilibrium of peptides in solution are to be pursued.

6) The distribution of antibodies to different determinants of Nase are to be correlated with the idiotypes of anti-Nase antibodies made by the several strains (in collaboration with G. Fathman of the NCI Immunology Branch).

Keyword Descriptors:

Immune response genes, staphylococcal nuclease, immunogenetics in mice, immunological determinants, electron paramagnetic resonance, hemoglobin, Freund's adjuvant.

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,015-01 LCB
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antibodies to an Amino Terminal Fragment of Hemoglobin S.

Previous Serial Number: none

Principal Investigators: John Curd
Neal Young
Alan N. Schechter

Man Years:

Total: 0.7
Professional: 0.7
Other: 0

Project Description:

Objectives: Previous investigations in this laboratory have described antibodies produced by immunization with the fifty amino acid carboxyl terminus of staphylococcal nuclease. These antibodies were employed in immunological studies of conformational changes of nuclease in solution. A similar approach was chosen for preparation of antibodies specific for the amino terminal fifty-five amino acid fragment of the beta globin of hemoglobin S. These antibodies are being used to study conformational changes in this region of hemoglobin S.

Methods and Major Results:

1) Preparation of amino terminal fragment. Hemoglobin S was isolated from blood of homozygous donors. The beta chains were isolated by treatment of hemoglobin S with p-chloromercuribenzoate and chromatography on carboxy methyl cellulose. Heme was removed from the beta chains by acid extraction with methyl ethyl ketone to yield beta globin. The globin was cleaved with cyanogen bromide in 70% formic acid and the fragments separated by gel filtration in 1M acetic acid. The amino terminal fifty-five amino acid sequence was characterized by its molecular mass, absorption at 280 nm, amino acid composition, and peptide map.

2) Antibodies were elicited in sheep by repeated immunization with the fragment in complete Freunds adjuvant. The antibodies form precipitation lines with beta globin and hemoglobin S in immunodiffusion.

3) A radioimmunoassay utilizing radioiodinated fragment and dextran-coated charcoal was developed. The antibodies have an affinity of 10^8 M^{-1} with the peptide. The antibodies also react with beta S globin, beta A globin,

hemoglobin S, and hemoglobin A as shown by competition with the radioiodinated fragment. The shape of the bound/free versus fragment concentration plots are unusually steep and suggest a "cooperative phenomenon" which is under further study.

Significance to Biomedical Research: These antibodies are specific for the amino terminus of β S globin. They can be used in studies of the immunochemistry of the fragment, β globin, and hemoglobin S. The antisera contain subpopulations which are specific for antigenic determinants present only on the S-fragment, S globin, and hemoglobins. These antibodies may be isolated and used as immunochemical reagents in diagnosis and screening.

Proposed Course of Project: The antibodies and radioimmunoassay are being used to study:

1) antibody interaction with polypeptides in solution with emphasis on "cooperative phenomena".
2) the influence of O_2, CO, and 2,3-diphosphoglyceric acid on the immunological properties of hemoglobin S.
3) the S-specific determinants present on the fragment, beta S globin, and hemoglobin S.

Keyword Descriptors:

Antibodies, sickle hemoglobin, immunochemistry, radioimmunoassay, hemoglobin, sickle cell anemia, protein conformation.

Honors and Awards: None

Publications: None

Project No. Z01 AM 25,016-02 LCB
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunochemical and Chemical Studies of Hemoglobin and its Derivatives.

Previous Serial Number: NIAMDD-LCB-15

Principal Investigators: Neal Young
John Curd
Ann Eastlake
Alan N. Schechter

Cooperating Units: Department of Medicine, State University of New York, Syracuse, New York (Dr. Bruce Furie)

Man Years:

Total: 2.4
Professional: 1.4
Other: 1.0

Project Description:

Objectives: Studies on the conformation of hemoglobin in solution in our laboratory have been pursued from two directions, both suggested by previous experience with staphylococcal nuclease. First, the interaction of antibody and antigen, the latter a complete hemoglobin molecule or fragment of hemoglobin, have been shown to be dependent on the conformation of the protein in solution. The focus of our interest in the hemoglobin molecule has been the amino terminal end of the beta chain, which contains the amino acid substitution of valine for glutamic acid at the sixth residue in sickle hemoglobin (hemoglobin S), and is also the binding area for 2,3 DPG and of importance in the conformational transition from the relaxed to tense state which underlies the cooperative change in structure accompanying oxygenation.

Second, studies on nuclease and other proteins have demonstrated that fragments of the protein, inactive alone in solution, may complement each other and incomplete protein molecules so as to restore structure and some activity. These observations suggested that polypeptides of similar sequence to portions of the individual chains of hemoglobin might interact with the whole molecule and alter its functional properties. The decreased solubility of hemoglobin S is currently accounted for by presumed interaction between the abnormal amino terminus and a complementary site located elsewhere on the molecule. Peptides similar to this amino terminal region might compete for such a site and block this interaction. Alternatively, peptides of sufficient size might participate in binding and serve as probes for the complementary site location.

The goal of this project has been to prepare conformationally specific antibodies and to study their interaction with hemoglobin and its fragments and to prepare peptides for complementation studies.

Methods Employed: Gamma globulin from goats immunized with hemoglobins A and S has been partially purified by ammonium sulfate precipitation. Antibody function has been assessed with a wide variety of immunological techniques, including quantitative precipitation, hemagglutination, complement fixation, and complement fixation inhibition. However, many of our preparations were of such low activity that a radioimmunoassay using competitive protein binding and a double antibody precipitation step was developed. Hemoglobins A and S were carbamylated with ^{14}C potassium cyanate as a tracer for this assay.

Large amounts of hemoglobin S have been prepared, both by ion exchange chromatography of units of AS blood and by lysis of units of SS blood. The minimal gelling assay, developed by Bookchin and Nagel, has been employed. This assay uses sickle hemoglobin at high concentrations, similar to the concentration of the protein in the erythrocyte, which is deoxygenated and then evaporated gradually until the phase change from liquid to solid occurs, reflecting the protein's decreased solubility in solution. The concentration at the point of phase change is determined. Recently, more quantitative and chemically defined methods of measuring hemoglobin S solubility have been devised in other laboratories (such as that of Eaton and Hofrichter), particularly kinetic assays which measure the delay time between deoxygenation and phase change by light scattering. These methods as well as the minimal gelling assay have been used to study perturbants of the gelling process. In addition, interaction in dilute solution has been assessed with more conventional chemical methods, such as equilibrium dialysis and affinity chromatography.

Fragments of the amino terminal end of beta S hemoglobin have been prepared using enzymatic digestion with trypsin and chymotrypsin and chemical cleavage with N-bromosuccinimide and cyanogen bromide. About a dozen smaller peptides, which center about the beta 6 valine, have been synthesized by the Corley-Sachs modification of the Merrifield method and subsequently purified.

Sequential immunoabsorptions have been performed with affinity chromatography and in batch procedures with hemoglobins fixed to ion exchange resins. A number of well characterized liganded species of hemoglobin have been prepared for immunological study.

Major Results: Antibodies specific to hemoglobins A and S have been raised in goats and fractionated with ammonium sulfate. These antibodies absorb to hemoglobin — Sepharose columns and may be eluted quantitatively with urea or high salt concentration but with much loss of antibody activity. Antibodies with specificity to the amino terminal end of β^S globin have been prepared by fractionation on Sepharose columns made with synthetic and proteolytic peptides. Specific antibodies have also been obtained with sequential immunoabsorption.

Hemagglutination, complement fixation, and quantitative precipitation was found to be relatively insensitive to the antigenic distinctions between various hemoglobins and a radioimmunoassay has been developed which is capable of quantitatively detecting such differences. Current studies with this assay include assessment of inhibition of antibody-antigen binding by small peptides, relative reactivity of various liganded states of hemoglobin, and examination of antibodies specific to hemoglobin S. These studies are paralleled in a second radioimmunoassay system utilizing antibodies raised against a fragment of β globin.

Interaction of sickle hemoglobin and small peptides has been approached in a variety of ways. The gelling assay has failed to reveal an effect on gellation of all the natural amino acids and a variety of peptides from the amino terminus of β-globin. Currently selected peptides are being examined in a more sensitive assay system developed by Eaton et al. which examines gelling kinetically.

Current studies include equilibrium dialysis and affinity chromatography in an attempt to demonstrate interaction with peptides. The interpretation of the results, so far, has been obscured by deviations from physico-chemical ideality.

Significance to Biomedical Research: The use of these methods to prepare antibodies with high specificity to individual hemoglobin types offers the possibility of developing radioimmunoassays for detection and quantitation of normal and abnormal hemoglobin species. This may have particular use in prenatal diagnosis of sickle cell disease, but could also replace the usual electrophoretic methods now used to determine hemoglobin types.

The use of these antibodies to study hemoglobin conformation offers a powerful new method to look for subtle conformational changes. The analysis of the interaction of peptides with hemoglboins offers a way of studying the surface structure of normal and mutant hemoglobins and the peptides may offer a method to affect gellation.

Proposed Course of Project: The collection and analysis of data from these two approaches to studying hemoglobin structure and function will be continued until definitive conclusions are reached.

Keyword Descriptors:

Hemoglobin, sickle hemoglobin, antibodies, radioimmunoassay, conformation.

Honors and Awards: None

Publications:

Hercules, J.I., Schechter, A.N., Eaton, W.A., and Jackson, R.E. (Eds.) Proceedings of the First National Symposium on Sickle Cell Diseases, U.S. Government Printing Office, Washington, D.C., 1975.

Serial No. Z01 AM 25,017-03 LCB
1. Lab of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Transfer Ribonucleic Acids and their Interactions with Proteins

Previous Serial Number: NIAMDD-LCB-16

Principal Investigators:
Dan Eilat
Paola Di Natale
Ken Thomas
Peter Schiller
Alan N. Schechter

Cooperating Units: Arthritis and Rheumatism Branch, NIAMDD (Dr. Fred Steinberg); Chemistry Branch, NCI (Dr. Dolph Hatfield); Istituto di Chimical Biologica, Universita di Napoli, Naples, Italy (Prof. Francesco de Lorenzo)

Man Years:
Total: 3.0
Professional: 3.0

Project Description:

Objectives:

1) To date, over sixty different tRNA molecules have been sequenced and the first crystal structure has been elucidated. The conformation of tRNA in solution, during the various stages of protein biosynthesis remains however to be investigated. One approach to this problem is to raise antibodies to tRNA which will be conformationally specific. These antibodies would probably distinguish between conformations of uncharged tRNA, aminoacyl-tRNA, peptidyl-tRNA, ribosome bound tRNA, etc. The finding of such an antibody could also contribute very much to the field of protein-nucleic acid interaction, since the stability of such a complex would be expected to be higher than that of many tRNA-enzyme complexes.

2) Regulation of tRNA biosynthesis has been implicated in procaryotic and eucaryotic cells following bacteriophage infection and oncogenic virus infection respectively. The MS2 virus-E. coli system provides a unique opportunity to observe this phenomenon, because the sequences of different viral proteins as well as many viral RNA sequences have lately been established. We undertook to look at the specific changes which occur within a few groups of isoacceptor tRNAs, before and after virus infection, and try to relate these changes to the coding properties of the viral messenger RNA.

3) The interaction between histidine and phenylalanine tRNA and their cognate synthetases are biologically important examples of tRNA-protein interactions and are being studied by a variety of methods. The synthetases from Salmonella have been purified and characterized and methods of specific labelling of the tRNA have been developed to facilitate the study of the interaction. More recently we have begun to study the interaction of synthetic peptides with tRNA.

Methods Employed: Four different conformations of E. coli tRNA (native tRNA, cross-linked native tRNA, heavily cross-linked native tRNA and cross-linked denatured tRNA) were prepared by reaction with formaldehyde at different temperatures, and in the presence or absence of magnesium. These tRNA's were used alone or in different complexes (spermine, methylated bovine serum albumin) for immunization of rabbits and mice.

Radioactive [^{3}H] tRNA was prepared by isolating tRNA from Q13 E. coli cells grown on [^{3}H] uridine containing medium.

The radioimmunoassay used for detection of antibodies included ammonium sulfate precipitation and sephadex chromatography of [^{3}H] tRNA-antibody complexes. Ribonuclease activity was removed from serum by charcoal treatment or by affinity chromatography. E. coli C-3000 were grown and infected with MS2 virus in the NIH fermenter unit. The tRNA was extracted from preinfected and postinfected cells, and the patterns of isoacceptors were determined by RPC-5 reversed phase chromatography.

The histidine and phenylalanine tRNA synthetases from Salmonella have been purified by ion exchange chromatography and by affinity chromatography on an amine acid resin developed here. Amino acid analysis, peptide mapping, absorption and fluorescence spectroscopy, ultracentrifugation and other methods have been used to characterize their molecular structure. The cognate tRNA has been labeled with the N-hydroxysuccinimide esters of Dansyl glycine and N-methylanthranilic acid to give a species with a specific fluorescent probe. The location of the reactive group has been established. The solid phase synthetic method has been employed to synthesize a hexapeptide which we believe will bind in a specific groove of the tRNA.

Major Findings:

1) Rabbits and mice produce small amounts of low affinity antibodies to the various structures of tRNA mentioned above. In all cases, higher levels of binding activity were found to denatured tRNA than to native tRNA. This could reflect an early denaturation of tRNA in the animal system. A high affinity binding activity to native tRNA was found in 8 month old NZB/WZW mice (which have an autoimmune disease, very similar to Systemic Lupus Erythematosus (SLE) in humans). This tRNA binding activity has a dissociation constant of about 2×10^{-8} M, and is very specific for native tRNA. Competition experiments have shown that complex formation is weakly inhibited by different nucleic acids and nucleic acid components such as nucleotides, synthetic single-stranded and double-stranded polynucleotides, and DNA. They are strongly inhibited by different conformations of tRNA in the order of their extent of denaturation. tRNA binding activity was also found in some S.L.E. patients.

2) tRNA specific for leucine was investigated before and after bacteriophage infection. The amino acid acceptance of $tRNA^{Leu}$ seemed to remain unaltered, but the isoacceptor tRNA distribution has been drastically changed. $Leu\text{-}tRNA_{II}^{Leu}$ which is present in negligible amounts in the uninfected cells becomes the major tRNA after infection. In addition, two chromatographically distinct new peaks of Leu tRNA appear after infection. The dramatic increase in $Leu\text{-}tRNA_{II}^{Leu}$ is compatible with the availability of its assigned codons in the MS2 message for the coat protein.

3) The molecular weight of the homogeneous phenylalanine Salmonella tRNA synthetase has been established by disc gel and ultracentrifugation methods as 220,000 with an α_2 β_2 subunit structure, with subunits of 86,000 and 36,000 molecular weight. The amino acid composition and various spectroscopic and immunochemical properties of the enzyme have been established. This complements parallel work on the histidine synthetase.

The location of the fluorescent group on the phenylalanine tRNA has been established as the "X-base" of the variable stem and this offers a new means of studying the conformation and interactions of tRNA with a specific probe on a region not accessible before. The interaction of this labeled tRNA with the synthetase is being studied. In addition a hexapeptide which is expected to be complimentary to the tRNA has been synthesized, with a radioactive label, and is being purified for binding studies.

Significance to Biomedical Research: The tRNA-antibody project may lead to new information on the origin, development, and genetics of certain autoimmune diseases, particularly Systemic Lupus Erythematosus. The tRNA regulation project will provide a better understanding of the cellular mechanisms following viral infection.

The development of antibodies to tRNA, and other nucleic acids, offers great potential for studying the conformation of nucleic acids (by methods analogous to those we have used for studying the conformation of proteins) and for probing various questions related to the structure and function of nucleic acids in the ribosome, nucleus and in several biological processes. The characterization of these NZB/NZW mice antibody proteins is a start in this direction.

The analysis of the interaction of several tRNA molecules with their cognate synthetases and with specific peptides will be used in the study of protein-nucleic acid interactions by physico-chemical techniques and may give information of the mechanisms of these biologically important phenomena.

Proposed Course of Project:

1) Further characterization of the tRNA binding activity in NZB/WZW mouse sera and in S.L.E. patient's sera will be done in order to show its antibody properties. Fractionation and partial purification by affinity chromatography will be attempted. The structure of tRNA during various stages of protein synthesis will be investigated by analysis of the specific interaction with the antibody. Further attempts will be made to raise antibodies in rabbits and other animals.

2) tRNA's specific for amino acids other than leucine will be looked at following virus infection in order to understand the regulation of tRNA biosynthesis.

3) The interaction of the phenylalanine tRNA with the peptides and synthetase will be studied by methods such as fluorescence spectroscopy, equilibrium dialysis, and nuclear magnetic resonance spectroscopy.

Keywords:

tRNA, amino-acyl tRNA synthetase, phenylalanine synthetase, nucleic acid antibodies, antibodies, viral infection, histidine synthetase.

Honors and Awards: None

Publications: None

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMICAL PHARMACOLOGY

I. PROTEIN STRUCTURE AND MECHANISM OF ENZYME AND HORMONE ACTION

Aminoacyl tRNA Synthetases

Over a period of years we have developed a hypothesis that the aminoacyl tRNA synthetases of yeast can exist in 2 forms. One is very labile and requires an activator. The other is more stable and works well without any activating substance, but it can be converted to the labile activator-requiring form by inorganic sulfide. New experiments with yeast suggest that both forms are present in the cells and that they vary in concentration in a reciprocal manner during growth: as the amount of one form increases, the other decreases, and vice versa. This cyclic process is accelerated by 100% oxygen and slowed by complete exclusion of oxygen. Further evidence indicates that this regulating mechanism may be a general one for all aminoacyl tRNA synthetases.

. . . . . Dr. S. Black and Mrs. B. Hazel

Studies Concerning the Mechanism of Exophthalmos and of Thyrotropin Receptor Function

We have demonstrated that thyrotropic hormone is exophthalmogenic but that partial pepsin digestion could yield an exophthalmogenic fragment of the thyrotropin molecule which has lost its thyroid stimulating ability. Studies concerning the binding of these hormonal factors and their biochemical effects on retro-orbital and thyroid tissues led us to suggest that malignant exophthalmos is a two-factor disease requiring the simultaneous presence of thyrotropin (TSH) or a TSH derivative without thyroid stimulating ability, and of an abnormal gamma globulin which is not a long acting thyroid stimulator (LATS) and which can be detected in the serum of patients with exophthalmos. The TSH or the TSH derivative is presumed to be the direct effector; the abnormal gamma globulin is presumed to target this effector by modifying its binding characteristics to retro-orbital receptors. The finding that cellular sensitivity (as measured by leucocyte migration assays) preceded the appearance of the abnormal serum gamma globulin has resulted in the use of this assay to detect Graves' disease patients who will get exophthalmos during treatment of their hyperthyroidism. This detection method and the use of nontoxic doses of Imuran have led to the prevention of this disease in a preliminary series of 600 patients.

Experiments reported this year have shown the following: (1) The structure of the EPF obtained from partial pepsin digests of bovine TSH. (2) Partial pepsin digestion can create an EPF derivative from human TSH. (3) EPF and TSH appear to act on retro-orbital tissue via an adenylate cyclase - cAMP system. (4) The structure of the TSH - EPF receptors on thyroid and retro-orbital tissue membranes is different. (5) The gamma globulin effect on retro-orbital tissue TSH - EPF receptors requires the presence of TSH or EPF, *i.e.*, alone it neither binds nor activates adenylate cyclase activity. (6) Trypsinization of thyroid cells in culture releases a 15,000-30,000 molecular weight TSH - EPF receptor fragment into the medium. This fragment can be pulse labeled and regrows when trypsinized cells are recultured. Coincident with regeneration of the receptor, cyclase stimulability by TSH returns. (7) TSH has a significant trophic effect on thyroid cells in culture. (8) The 15,000-30,000 molecular weight TSH - EPF receptor from bovine thyroid membranes has been purified to near homogeneity, and its functional and physical properties preliminarily studied. (9) In Belgium, Dr. Roger J. Winand has extended the clinical trial concerned with the prevention of exophthalmos.

. . . . . Drs. L. D. Kohn, R. L. Tate, B. R. Mullin, and R. J. Winand (University of Liege, Liege, Belgium)

Studies Concerning the Biosynthesis of Collagen

Type III procollagen has been purified from amniotic fluid. A specific antiserum has been developed to its precursor peptide portion which cross reacts with human type III procollagen. The antiserum has been used to show increases in type III procollagen in osteogenesis imperfecta and certain liver diseases.

A type I procollagen radioimmunoassay has been developed for use in biosynthesis experiments as well as for testing patients' sera.

. . . . . Drs. G. Lee, R. L. Tate, L. D. Kohn, and G. R. Martin (D:LB)

Studies Concerning Membrane Structure and Function

Active transport of many solutes by *Escherichia coli* membrane vesicles is coupled to the oxidation of D-lactate to pyruvate. This oxidation is catalyzed by a membrane-bound D-lactate dehydrogenase (D-LDH) which functions in the absence of pyridine nucleotides. When solubilized and purified to homogeneity, D-LDH has a molecular weight of approximately 75,000, contains 1 mole of flavin adenine dinucleotide per mole of enzyme, and is inactivated by 2-hydroxy-3-butynoic acid, a suicide substrate of the enzyme.

An antiserum specific for D-LDH has been described and characterized. Data have been presented which demonstrate that vesicles prepared from *E. coli* ML 308-225*dld-3* contain an enzymatically inactive but antigenically recognizable D-LDH molecule. Last, the antiserum has been used to show that vesicle preparations are not partially inverted, that native vesicles have the D-LDH located on their inner surfaces, and that D-LDH when bound to the outer surface of reconstituted mutant vesicles can drive transport. In these same studies ATPase is shown to be a poor marker of vesicle sidedness and the respiratory chain is shown to be stimulable to drive transport from either side of the membrane.

. . . . . Drs. L. D. Kohn and H. R. Kaback (Roche Institute of Molecular Biology, Nutley, New Jersey)

Studies Concerning Structure and Function of Oxido Reductases

Oxido reductases which convert glyoxylate, hydroxypyruvate, tartrate, and malate to glycolate, D(-)-glycerate, oxaloglycolate, and oxaloacetate, respectively, have been purified to homogeneity and characterized. Experiments have been directed towards understanding their mechanism of action, the mechanism by which they achieve specificity, and the mechanism by which their subunits associate to yield active enzyme. Present experiments continue to involve sequence procedures of the active site of the induced and constitutive hydroxypyruvate reductases.

. . . . . Dr. L. D. Kohn

Studies Concerning Centrifugation

As a cooperating unit, centrifugation studies have been performed for NIAMDD personnel in this and other laboratories, as well as personnel in other NIH and non-NIH research facilities.

A minicomputer hookup has been developed to expedite analysis of centrifugal data.

The computer has been extended in its usefulness to analyses of kinetic data from an online spectrophotometer and to analyses of dual label data from scintillation counters.

. . . . . Drs. R. L. Tate and L. D. Kohn

Tryptophan Synthase of Escherichia coli

Tryptophan synthase is a multienzyme complex with an $\alpha_2\beta_2$ structure. Recently developed methods for the preparation and crystallization of the $\alpha_2\beta_2$ complex and the component α and β_2 subunits have allowed us to initiate systematic comparative studies of the properties of the separate and reassociated subunits. These experiments are directed at understanding how the subunits interact and how subunit interaction changes the environment of amino acid residues in the active sites and interaction sites of the α and β_2 subunits.

Changes in the environment of aromatic amino acids which result from the association of the α and apo β_2 complex have been evaluated by difference absorbance spectra, circular dichroism spectra, and by chemical modification with N-acetylimidazole. The association of 2 α subunits with 1 apo β_2 subunit results in the transfer of 6 to 7 tyrosine residues from an exposed environment into a hydrophobic environment. Subunit association is also accompanied by very large changes in the circular dichroism spectrum in the aromatic region which appear to be due to changes in the environment of tyrosine residues and not in that of tryptophan residues.

Reactions of the β_2 subunit and $\alpha_2\beta_2$ complex with substrate analogs which may also be irreversible inhibitors are being compared. α-Cyanoglycine inhibits both the β_2 subunit and the $\alpha_2\beta_2$ complex. Our finding that the chromophoric, α-cyanoglycine derivative of the $\alpha_2\beta_2$ complex is much more stable than the corresponding derivative of the β_2 subunit indicates that the environment of the derivative is quite different in the $\alpha_2\beta_2$ complex.

Similar conclusions are being reached from comparative studies of derivatives of pyridoxal-P bound by the β_2 subunit and $\alpha_2\beta_2$ complex. Oxime and phenylhydrazone derivatives of the holo $\alpha_2\beta_2$ complex are tightly bound, whereas the corresponding derivatives of the holo β_2 subunit are readily removed. Comparative circular dichroism studies of derivatives of the β_2 subunit and $\alpha_2\beta_2$ complex are in progress.

. . . . . Drs. E. W. Miles, O. Adachi, and M. Moriguchi

Urocanase

Studies have been carried out on the isolation and characterization of the keto-compound that appears to be a cofactor of this enzyme, and bound to it in covalent linkage.

. . . . . Dr. A. T. Phillips (Guest Worker, LBP; Professor of Biochemistry, The Pennsylvania State University)

II. SYNTHESIS OF PROTEINS AND NUCLEIC ACID

Nucleic Acid Synthesis

Replication of linear duplex T4 DNA *in vivo* requires several proteins encoded by the phage DNA including the T4 DNA polymerase, the T4 DNA unwinding protein (32 protein), and the proteins encoded by genes 44, 62, 45, and 41. We have developed a complementation assay, based on the ability of a protein to stimulate DNA synthesis using an endogenous template in a crude lysate of cells infected with phage defective in that protein, to monitor the purification of the proteins encoded by genes 44, 62, and 45. The protein purified to apparent homogeneity using gene 44 complementation as an assay is actually a complex of the gene 44 and 62 proteins; gene 62 complementation activity and a DNA dependent ATPase (and dATPase) follow gene 44 complementation activity during the purification, in agreement with the results of Alberts and coworkers for the 44-62 protein purified by a different procedure. The complex does not have DNA polymerase, DNA exo- or endonuclease, or RNase H activity. We have also purified the gene 45 protein to apparent homogeneity and have shown that it does not have DNA polymerase, DNA nuclease, DNA dependent or independent ATPase, or RNase H activity. The 45 protein is of special interest because in addition to its role in DNA replication, it is required for late mRNA synthesis (Geiduschek) and binds to columns containing the T4 modified RNA polymerase (Ratner). The purification of the gene 41 protein is in progress.

T4 DNA polymerase by itself is unable to copy a nicked duplex DNA template *in vitro*, apparently because it is unable to displace the DNA strand which is annealed to the DNA strand serving as the template. We have previously shown that under conditions of low Mg^{2+} and low salt, where the DNA helix is less stable, the addition of 32 protein to the T4 polymerase permits limited synthesis with a nicked duplex template, giving renaturable DNA as the principal product. At higher salt and Mg^{2+} concentrations, we have now shown that synthesis with this template occurs only if the gene 44-62 and gene 45 proteins are present in addition to the polymerase and DNA unwinding protein. The rate of this reaction increases with increasing concentrations of each of these proteins, and the product is not rapidly renaturable. Under these same conditions, the rate of synthesis of the polymerase with single stranded DNA templates is greatly stimulated by the gene 44-62 and gene 45 proteins and does not require the 32 protein.

. . . . . Dr. N. G. Nossal

We have continued our study of the T4 DNA polymerases purified from cells infected with phage having temperature sensitive mutations in the structural gene for the polymerase which at permissive temperatures have an increased (mutator) or decreased (antimutator) frequency of mutation in other genes. We have previously used the homopolymer pair, poly dA · poly dT, to study the frequency of *in vitro* copying errors and have shown that the noncomplementary nucleotides, dGTP and dCTP, are utilized more frequently by the mutator (*ts* L88) and less frequently by the antimutator (*ts* CB120) than by the wild-type enzyme. Utilization is measured by the DNA dependent production of nucleoside monophosphate from triphosphate, since the noncomplementary residues are removed so efficiently by the exonuclease activities of each enzyme. Using appropriate terminally labeled polymers, we have now shown that the rate of nucleoside monophosphate production in this system is not limited by the rate at which the enzymes can hydrolyze the 3' terminal complementary or noncomplementary residues. Thus the enzymes appear to differ in the frequencies with which they incorporate noncomplementary nucleotides. In addition, they may have differences in the efficiency of "editing" by nuclease removal of incorporation errors, which cannot be evaluated in this system.

We have also shown with this homopolymer pair that the wild-type enzyme has a markedly higher apparent K_m for noncomplementary than for complementary nucleotides. Both the mutator and antimutator enzymes have apparent K_m's for both complementary and noncomplementary nucleotides which are similar to those of the wild-type enzyme. The mutant enzymes showed interesting differences when their apparent K_m's for polymer were compared using a fixed concentration of either complementary or noncomplementary deoxynucleoside triphosphates. With the complementary nucleotide, the wild-type and antimutator enzymes had similar "K_m's" for polymer, while that of the mutator enzyme was markedly increased. When the "K_m" for polymer was measured by utilization of noncomplementary nucleotide, the "K_m" of the mutator increased, that of the antimutator decreased, while that of the wild-type was similar to the "K_m" measured for the same enzyme with the correct nucleotide.

Mn^{2+} has been shown by others to be a mutagen for T4 phage *in vivo*. We have now shown that Mn^{2+} also increases errors made by the polymerases *in vitro*, both with the homopolymer pair discussed above and with the alternating polymer, poly[d(A-T)]. Synthesis with the latter template, which has extensive intrastrand hydrogen bonding, is stimulated by the gene 32 DNA unwinding protein, and the addition of this protein decreases the frequency of errors to a small but significant extent.

. . . . . Drs. F. D. Gillin and N. G. Nossal

Most enzymes required for *Escherichia coli* DNA replication have been identified by mutations in bacteria which are temperature sensitive for DNA synthesis or for the joining of short DNA pieces. We think that other enzymes required for DNA replication may be recognized by their effect on the fidelity of DNA replication. We have therefore initiated a search for *E. coli* mutants which have an increased frequency of mutation and are temperature sensitive for growth. For this purpose we have developed a method suitable for screening large numbers of clones in which lac$^+$ mutants arising in a lac$^-$ colony are identified as white papillae on the surface of red colonies growing on tetrazolium plates. We have shown that several *E. coli* mutator mutants, isolated by others by different procedures, are easily identified by the large number of lac$^+$ papillae arising in lac$^-$ colonies with the mutator phenotype. We are now using this procedure to search for new mutator genes.

. . . . . Drs. E. B. Konrad and N. G. Nossal

Elongation Factors

Recently work in this laboratory has been directed to studying the means by which cells regulate their levels of elongation factor Tu. By 3 independent means, we have shown that bacteria growing at very different growth rates contain 5 to 7 molecules of Tu per ribosome. These data are in contrast with those found by others for the elongation factors Ts and G, which are present at one copy each per ribosome. The question arises as to why does the cell require so much Tu, and efforts are now underway in an attempt to answer it. Data obtained earlier in this laboratory suggested that Tu could exist as an aggregate, and it is possible that in protein synthesis Tu functions as a polymer. It is also possible that Tu has functions in the cell other than those of protein synthesis.

. . . . . Dr. A. V. Furano

Methylation of Amino Acids

We have devised a new method for the simple analysis of methylated amino acids based on autoradiography. With this technique a survey of protein methylation in a prokaryote *Escherichia coli* and a eukaryote, fibroblasts in culture, was carried out in an attempt to identify, quantitate, and determine the subcellular localization of all the methylated amino acids found in the protein of these organisms.

In mammalian cells using an established mouse fibroblast line (3T3), we have found that nuclei-free and mitochondria-free cytoplasm contain at least 4 identifiable methylated amino acids: $N^{\epsilon},N^{\epsilon}$-dimethyllysine, $N^{\epsilon},N^{\epsilon},N^{\epsilon}$-trimethyllysine, N^{G},N^{G}-dimethylarginine (or N^{G}-methylarginine), and N^{G},N'^{G}-dimethylarginine. The crude nuclear pellet also contains 4 identifiable methylated amino acids, but in contrast to the cytoplasmic fraction, N^{ϵ}-methyllysine is present and N^{G},N^{G}-dimethylarginine is absent. Qualitatively, the nuclear methylated amino acids were identical to those found in purified nuclear histones.

The ribosomal subunits of the mammalian cells contained only small amounts of the methylated amino acids; the 40S subunit contained a substantial amount of just one, N^G,N^G-dimethylarginine (or N^G-methylarginine), and smaller amounts of N^G,N'^G-dimethylarginine, and an as yet unidentified methylated amino acid. The 60S subunit contained even smaller amounts of methylated amino acids, 50% of which was $N^\varepsilon,N^\varepsilon,N^\varepsilon$-trimethyllysine and smaller amounts of N^ε-methyllysine, $N^\varepsilon,N^\varepsilon$-dimethyllysine, and N^G,N'^G-dimethylarginine.

These results were in marked contrast to those that we obtained with the prokaryote, *E. coli*. Only the proteins of the 50S ribosomal subunit of the bacteria contained methylated amino acids. Of those present 50% was $N^\varepsilon,N^\varepsilon,N^\varepsilon$-trimethyllysine, with the remainder distributed about equally between N^ε-methyllysine and 3 unknowns. All of the N^ε-methyllysine was apparently in the small acidic proteins, L7 and L12.

In addition, the autoradiographic method has allowed us to detect at least 5 new minor but yet unidentified methylated amino acids, 3 in *E. coli*, and one of these and 2 others in mouse fibroblasts.

. . . . . Drs. A. V. Furano and M. Klagsbrun (now at Children's Hospital Medical Center, Boston, Massachusetts)

III. COMPLEX CARBOHYDRATES

Antibodies produced by immunization with purified oligosaccharides coupled to polylysine specifically bind complex carbohydrates containing the same nonreducing terminal sugar sequence. For example, goat antibodies against lacto-*N*-difucohexaose I (a human Le^b blood group hapten) react specifically with human $Le(a^-b^+)$ erthrocytes and with soluble Le^b blood group substance. Antibodies produced in rabbits against lacto-*N*-fucopentaose II (a human Le^a blood group hapten) exhibit similar Le^a specificity. In addition, these antibodies specifically bind the corresponding free sodium borotritide-reduced haptens.

Both antibodies can be purified by affinity chromatography on Sepharose substituted with the appropriate oligosaccharide. Affinity columns were prepared by reacting cyanogen bromide-activated Sepharose with derivatives of the oligosaccharides coupled to β(p-aminophenyl)ethylamine as previously described. Radioimmune assay systems have been developed using these antibodies and the corresponding labeled haptens. The assays can detect 0.01 nanomoles of the respective nonreducing sugar sequences in glycoproteins and glycolipids.

. . . . . Drs. D. A. Zopf and V. Ginsburg

IV. CELL SURFACES

Structure and Assembly of Escherichia coli Membranes

The surface of *E. coli* consists of an inner or cytoplasmic membrane, a rigid cell wall, and an outer membrane. The outer membrane is assembled from molecules made elsewhere in the cell, and for this reason and because of its relative simplicity, serves as a good model for studying membrane assembly.

Previous work from this laboratory and others indicated that there were about 10-50 specific insertion points in the outer membrane into which newly synthesized lipopolysaccharide was translocated from the inner membrane, and from whence it spread to the remainder of the outer membrane. Using a mutant which changes the antigenic specificity of its lipopolysaccharide when galactose is added to the membrane, we purified antibody specific for this lipopolysaccharide and are using it to isolate the insertion points. Cells are grown in the absence of galactose and galactose added at 0 time. A few seconds later the cells are harvested and their membrane isolated and fragmented. Membrane fragments that specifically contain newly inserted lipopolysaccharide are separated, either by precipitation with antibody specific for the new lipopolysaccharide or by fractionation on columns containing Sepharose linked to this antibody. So far we have defined the specificity of the membrane reaction with antibody and are currently working to minimize nonspecific trapping of membrane fragments which is necessary to achieve the isolation of a pure preparation of insertion points.

. . . . . Dr. L. Leive

The outer membrane appears by several criteria to have true membrane (lipid-protein bilayer) character, but several reports using electron spin resonance, x-ray diffraction, fluorescent probes, and other methods have suggested that it may differ in having at least some regions in which lipid mobility is restricted. It has been suggested that the lipopolysaccharide creates rigid regions by some mechanism, but there is no evidence as yet bearing on this hypothesis. Since treating cells briefly with EDTA releases half of the outer membrane lipopolysaccharide, we are beginning experiments to determine whether such EDTA treated outer membranes differ from normal outer membranes in having more regions accessible to nitroxide labeled fatty acid probes as analyzed by electron spin resonance.

. . . . . Drs. L. Leive and S. Rottem (Division of Blood and Blood Products, Bureau of Biologics, NIH)

Although EDTA treatment of *E. coli* results in release of half of the outer membrane lipopolysaccharide, electron microscopy of thin sections or negatively stained preparations revealed no differences in morphology of the membrane. However, we have now been able to demonstrate a difference in the ultrastructure of the outer face of the outer membrane after such treatment using freeze-etch techniques. Areas of the membrane also appear thinner, and sharply differentiated from thicker "plateaus" that remain in the membrane.

. . . . . Drs. L. Leive and M. E. Bayer (Institute for Cancer Research, Philadelphia, Pennsylvania)

Isolation of a New E. coli Transducing Phage

E. coli 0111, which we have used for several years for study of the outer membrane because of its well defined lipopolysaccharide, has been reported occasionally in the literature to carry a phage-like particle. This particle was reported to kill, but not multiply in, other strains of *E. coli*. We have now analyzed this particle and have been able to make it multiply at low efficiency on other strains. We have also demonstrated that it can transduce markers from *E. coli* 0111 to other strains, indicating that it is a transducing phage, possibly defective. The relationship of this phage to other known transducing phages is currently being determined.

. . . . . Drs. L. Leive and W. G. Coleman, Jr.

Mutants of E. coli Defective in Lipid Synthesis

A new method has been developed which permits the rapid screening of *E. coli* colonies for mutants in enzymes of phospholipid metabolism. The screening technique is termed replica printing and should be applicable not only to phospholipid metabolism but also to nucleic acid and protein synthesis.

Four mutants were identified with low levels of CDP-diglyceride : *sn*-glycero-3-P phosphatidyl transferase (phosphatidylglycerophosphate synthetase) in extracts. With a similar assay, 10,000 additional colonies were screened for mutants in CDP-diglyceride : L-serine phosphatidyl transferase (phosphatidylserine synthetase), and 4 strains were found in which the enzyme was thermolabile.

. . . . . Dr. C. R. H. Raetz (Guest Worker, LBP; Research Associate in Pharmacology, NIGMS, NIH)

V. MECHANISMS OF INHERITANCE IN SACCHAROMYCES CEREVISIAE

The "Killer Character" of Saccharomyces cerevisiae

Strains of *S. cerevisiae* carrying a 1.5×10^6 molecular weight double-stranded RNA plasmid secrete a toxin which is lethal to strains not carrying this plasmid.

We have isolated mutants in chromosomal genes needed to replicate or maintain the killer plasmid (*pets*, *mak1*, *mak2*, *mak3*) and other mutants in chromosomal genes needed for *killer expression* (*kex1*, *kex2*). A third class of mutants are defective in the cytoplasmic genome.

We have located *mak1* near the centromere of chromosome XV while *mak3* is near *tyr7* on chromosome XVI. *Kex2* is on chromosome XIV, not linked to any previously known markers on this chromosome. *Pets* is between the centromere and mating type locus on chromosome III.

Mutants assigned to *kex2* by complementation show reduced sporulation ability (> 200-fold) in homozygous diploids. *Kex2* haploids also show an α-specific mating defect, *i.e.*, *α kex2* strains mate with *a* strains at a very low frequency, but *a kex2* strains mate with *α* strains normally. Normal *α* strains secrete an oligopeptide, "*α* mating hormone," which prepares normal *a* cells for mating. *Kex2* strains of the *α* mating type which fail to mate, fail to secrete this hormone, but *a kex2* strains, which mate normally, respond normally to the hormone produced by normal *α* strains. The hormone defect in *α kex2* cells, while sufficient to account for their inability to mate, is not the only defect, since supplying active "*α* hormone" does not increase their mating ability.

Thus, mutation of a single chromosomal gene, kex2, results in 3 defects: inability of α cells to mate, inability of homozygous diploids to undergo meiosis and sporulation, and a defect in expression of killing.

The pets gene is needed to maintain and replicate both the killer plasmid and another cytoplasmic genetic element, mitochondrial DNA. We have recently shown that this gene is also needed for normal mitotic growth, since 2 independent mutations in this gene result in temperature sensitivity for growth which co-segregated with the other defects, which are expressed even at the permissive temperature for growth.

Mutation of the mak1 gene can also result in temperature sensitivity for growth, with the defect in killer plasmid replication again seen at the permissive temperature for growth. Temperature-resistant revertants have simultaneously regained the ability to maintain the killer plasmid.

Sek Mutants. Mutants in at least 2 new chromosomal genes have been isolated which are needed for sensitivity to the killer toxin. The mutant phenotype (resistance) is recessive to the wild-type phenotype (sensitivity). Such mutants are easily isolated as the main class of survivors of a sensitive strain after co-cultivation with a killer strain.

Plasmid Mutants Dependent on Host Diploidy. Mutants of the killer plasmid have been isolated which have become dependent upon chromosomal diploidy for the expression of plasmid functions and for replication or maintenance of the plasmid itself. These mutants show no evidence of defects in any chromosomal gene needed for expression or replication of the killer plasmid.

. . . . . Drs. R. B. Wickner and M. J. Leibowitz (Guest Worker, LBP; Research Associate in Pharmacology, NIGMS, NIH)

VI. POLYAMINES

Glutathionylspermidine accumulates in *Escherichia coli* at the end of growth, and accounts for all of the intracellular spermidine and one-half of the intracellular glutathione. Added [^{35}S]glutathione or [^{3}H]spermidine are incorporated into glutathionylspermidine. Glutathionylspermidine has been isolated from *E. coli* and characterized by chemical and enzymatic techniques. Turnover studies have shown that, even when it is accumulating, glutathionylspermidine is in equilibrium with unbound glutathione and with free spermidine. Enzymes are present in *E. coli* for both the synthesis and the degradation of glutathionylspermidine.

Closely related to these studies are our studies showing that glutathione is normally present in large amounts in *E. coli*. For this purpose, a new analytical technique was developed for the automated amino acid analyzer, based on elution from an anion exchange resin. This technique permits the quantitative determination of free and total glutathione, and of related compounds, in liver and other tissues, as well as in *E. coli*.

. . . . . Drs. C. W. Tabor and H. Tabor

The synthesis of adenosylmethionine is the first step in the biosynthesis of spermidine. The enzyme, which carries out this reaction in the presence of ATP, K^+, Mg^{2+}, and L-methionine, has been purified from *E. coli*. Mutants with low levels of this enzyme have been isolated by taking advantage of their resistance to ethionine, and have been shown to map at the met K locus. A mutant, which maps in the met K locus, and which is only ethionine resistant at elevated temperatures, was isolated, and shown to contain a temperature-sensitive adenosylmethionine synthetase, indicating that this is the structural gene for the enzyme.

. . . . . Drs. E. W. Hafner, C. W. Tabor, and H. Tabor

VII. EVOLUTION

Previous experiments on the origin of the genetic code indicated that 5-isobutyluracil has a significantly greater affinity for a peptide containing leucine than for analogous peptides with phenylalanine and valine. The implication was that the isobutyl side chain of leucine interacts with some specificity with the corresponding group of the uracil derivative, so that the latter may be a "primitive codon" for leucine. This type of experiment has now been extended to uracil derivatives with 5' side chains corresponding to the side chains of methionine [5-(methylthioethyl)uracil] and valine [5-(isopropyl)uracil]. A number of sulfur-containing analogs of methylthioethyluracil have also been made. The results of tests made thus far are encouraging to the hypothesis that a "like for like" pairing of side chains between peptides and primitive nucleic acids could have been the basis of the original genetic code.

. . . . . Drs. S. Black and H. M. Chao

VIII. TRAUMATIC SHOCK AND CELLULAR IMMUNITY

Experiments have shown that one effect of thermal injury on cellular immunity is to diminish the cytotoxic ability of lymphocytes from spleen, lymph nodes, and peripheral blood. A factor in serum collected from burned mice, which appeared to be a protein, was found to inhibit cytotoxicity of lymphocytes.

Fluid accumulated in the mouse tail subjected to a vacuum was shown to decrease 70% during the first 5 months of life. There was a close correlation between transvascular fluid movement and growth rate.

. . . . . Drs. K. Markley and S. M. Rosenthal and Mrs. E. T. Smallman

IX. MYCOBACTERIUM

Studies have been continued on our new method for the cultivation of granulocytic whole blood cells.

Cytochemical studies of the natural feeder cell for the mouse granulocytes revealed that the feeder cell was probably related in some way to the reticular cell of the bone marrow.

Motion picture studies were made on the free movement of human blood polymorphonuclear leucocytes leading to the proposal of a term of "mobility coefficient" for the leucocytes.

Good growth of macrophages was observed in cultures obtained from the following tissues: blood leucocytes, alveolar washings, subcutaneous tissue, lungs, bone marrow, spleen, thyroid, thymus, and heart muscle.

Motion picture studies on the so-called "novel cell," a dendritic cell of the spleen, revealed a survival of these cells for a period of 23 days in comparison with only a few hours reported by others.

A technique was developed for taking low-magnification motion pictures for the movement of cells out of a capillary tube as employed in the migration inhibitory factor (MIF) assay for human leucocytes and mouse peritoneal exudate cells.

A simple technique was developed for copying a 16 mm movie film using the laboratory microscope attached with a 35 mm camera back.

Two new phenazine compounds were studied for their chemotherapeutic action in mice in curing murine leprosy.

. . . . . Dr. Y. T. Chang

Project No. Z01 AM 23,140-17 LBP
1. Biochemical Pharmacology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biochemistry of Sulfur-Containing Compounds

Previous Serial Number: NIAMDD-LBP-1

Principal Investigator: Dr. Simon Black

Other Investigators: Dr. Helen M. Chao and Mrs. Blondel Hazel

Cooperating Units: None

Man Years:

Total:	4.2
Professional:	3.0
Others:	1.2

Project Description:

I. Protein Synthesis. The aminoacyl tRNA synthetases of all living organisms catalyze the following general reaction:

$$\text{amino acid} + \text{ATP} + \text{tRNA} \rightleftarrows \text{aminoacyl tRNA} + \text{AMP} + \text{PP}$$

We have found previously that a number of these enzymes from several sources are sensitive to inorganic sulfide, their activity in the presence of this agent becoming completely dependent upon activator-like substances such as an alkali-denatured protein and glutathione. In the past year we have shown that within the yeast cell 2 forms of the glycyl tRNA synthetase co-exist, one being sulfide-sensitive and the other equivalent to the sulfide-treated form. Of special interest is new evidence that these 2 forms seem to be interconverted in the course of cell growth, for each rise in the concentration of one is accompanied by a fall in the other. This cyclic interconversion is amplified and accelerated by an increase in the supply of oxygen, and diminished by anaerobiosis.

On the basis of their sensitivity to sulfide there is reason to believe that many, and perhaps all, of the aminoacyl tRNA synthetases of many species are subject to the same oxygen-modulated regulation as the glycyl tRNA synthetase of yeast. This mechanism thus promises to be a vital one in protein synthesis and could prove to be of crucial importance in understanding disease processes.

We plan to continue this study with the general objective of unraveling and illucidating the detailed components and operations of the mechanism by which the primary step of protein synthesis is regulated.

II. Evolution. Earlier experiments with several uracil derivatives, substituted with alkyl groups in the 5' position, indicated that 5-isobutyluracil has a greater affinity for a leucine-containing peptide than 5-phenyluracil, 5-benzyluracil, or 5-isopropyluracil. The isobutyl group of leucine was therefore indicated to interact with some degree of specificity with a like group on uracil. This type of experiment has now been extended to include peptides of the 5 amino acids of the first (uracil) column of the genetic codon table, phenylalanine, leucine, isoleucine, methionine, and valine. Uracil derivatives have been obtained or prepared having 5' side chains corresponding to the side chains of these amino acids. Encouraging experimental support has been obtained for the hypothesis that the first primitive genetic code was based on a "like for like" attraction between amino acid and uracil side chains of this kind. In particular, 5-(methylthioethyl)uracil and 5-isopropyluracil seem to have considerable affinities for peptides of methionine and valine, respectively.

Another project in this area involves the purification of an enzyme from *Erwinia herbicola* that catalyzes a phosphorylation of nucleosides by p-nitrophenyl phosphate. It is hoped to use this enzyme for the preparation of nucleotides of "primitive codons," eventually to link these together as primitive nucleic acids. The enzyme has been obtained in a reasonable state of purity and is presently being characterized more fully.

Keyword Descriptors: aminoacyl tRNA synthetase regulation - glycyl tRNA synthetase regulation - regulation of protein synthesis - origin of genetic code - evolution of genetic code.

Honors and Awards: None

Publications: None

Project No. Z01 AM 23,230-25 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemotherapy of Mouse Leprosy

Previous Serial Number: NIAMDD-LBP-2

Principal Investigator: Dr. Yao Teh Chang

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.9
Professional:	1.0
Other:	0.9

Project Description:

Growth of Macrophages Obtained from Various Sources. Good growth of macrophages was observed in cultures of the following mouse tissues: blood leucocytes, alveolar washings, subcutaneous tissues, lungs, bone marrow, spleen, thyroid, thymus, and cardiac muscles. These results suggest that similar techniques could be used to grow human macrophages of various tissues. This may be done by utilizing surgical materials discarded from the operation room of hospitals. At present, only the blood, bone marrow, and skin window are used for studies of human macrophages.

The Feeder Cell of Granulocytes as the Reticular Cell. Cytochemical studies of the "natural feeder cell" for mouse granulocytes revealed that the feeder cell showed not only the properties of macrophages, *i.e.*, phagocytosis, cytophilic antibodies, ferritin deposition and silver impregnation, etc., but also the alkaline phosphatase activity. This suggested that the feeder cell might be related in some way to the reticular cell of the bone marrow.

A Motion Picture of the Growth of Mouse Granulocytes. A 400-foot, 16 mm movie on the growth of mouse granulocytes was made. Multiplication and maturation of granulocytes were observed on top of the "natural feeder cells." Four successive divisions of a single precursor, presumably the promyelocytes, was filmed. A total of 3 crops of granulocytes was observed in a period of 3 weeks. This is the first movie showing the maturation of granulocytes in cell cultures.

This movie was presented at the American Society of Hematology. The movie was borrowed by Dr. D. H. Cormack, Division of Histology, University of Toronto, and video taped for his teaching purposes.

Growth of the "Novel Cell." Motion picture studies on the so-called "novel cell," a dendritic cell of the spleen, revealed a survival of these cells for a period of 23 days in comparison with only a few hours reported by others.

Copy of 16 mm Movie Film with the Laboratory Microscope. A simple method was developed for copying 16 mm movie films using the laboratory microscope equipped with an 1X objective and a 35 mm camera back. This method is convenient and time-saving and eliminates the necessity of using an expensive copying apparatus or the cutting frames off the movie reel for enlargements.

"Mobility Coefficient" of Leucocytes. In cooperation with Dr. Ralph J. Nossal, Physical Sciences Laboratory, Division of Computer Research and Technology, NIH, a motion picture study of the free movement of human polymorphonuclear leucocytes was made. A "mobility coefficient" of leucocytes was proposed. This offers a model for measuring the random movement of leucocytes which is important in the study of the migration inhibitory factor (MIF) of leucocytes.

The MIF Studies for Burned Mice and Hyperthyroid Patients. In cooperation with Drs. Kehl Markley and Brian R. Mullin of this laboratory, the MIF assay was made by taking motion pictures of cells migrating out of capillary tubes. A technique was developed for taking very low magnification movies, by using an 1X objective and a 5X eyepiece with a total of 5X magnification. This technique offers an accurate study of the dynamics of cell migration in comparison with the current method in which only the 24 hour migration is evaluated.

The Colony Stimulating Factor (CSF) for Granulocytes. In cooperation with Dr. Helen M. Chao of this laboratory, the effect of the most potent CSF of Metcalf, the endotoxin-treated mouse serum, on the growth of granulocytes is being studied in our cell model.

New Phenazine Compounds for Murine Leprosy. In cooperation with Dr. Norman E. Morrison, Johns Hopkins School of Hygiene, the effect of 2 new phenazine compounds, M9 and M28, is being studied in murine leprosy.

Keyword Descriptors: macrophages - granulocytes - chemotherapy of murine leprosy

Honors and Awards: None

Publications:

Chang, Y. T., and Andersen, R. N.: Cultivation of mouse bone marrow cells. Cytochemical studies of the granulocyte colony feeder cells. J. Reticuloendothel. Soc., 1975, in press.

Chang, Y. T., and Andersen, R. N.: Studies on the long-term growth of macrophages obtained from various sources (with presentation of a motion picture). In Wagner, W.-H., and Hahn, H. (Eds.) and Evans, R. (Co-Ed.): *Activation of Macrophages. Proceedings of the Second Workshop Conference Hoechst, Schloss Reisensburg, 25-26 October, 1973*. International Congress Series No. 325. Amsterdam, The Netherlands, Excerpta Medica, 1974, pp. 5-14.

Project No. Z01 AM 23,580-12 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein Synthesis in *Escherichia coli*

Previous Serial Number: NIAMDD-LBP-7

Principal Investigator: Dr. Anthony V. Furano

Other Investigators: None

Cooperating Unit: Dr. Michael Klagsbrun, Children's Hospital Medical Center,
Boston, Massachusetts

Man Years:

Total:	1.1
Professional:	1.0
Others:	0.1

Project Description:

Objectives:

To study the mechanism and control of protein synthesis. This laboratory is also involved in studying the processes and effects of protein modification (*e.g.*, methylation) in collaboration with Dr. Michael Klagsbrun, Children's Hospital Medical Center, Boston.

Methods Employed:

This laboratory has purified the peptide chain elongation factors present in *E. coli* and thereby devised methodology to measure directly the levels of these factors in these bacteria under varying conditions of growth. Studies are also being carried out on the elongation factors *in vitro*.

Protein Modification (in collaboration with Dr. M. Klagsbrun). We are identifying the types of methylated amino acids present in the various subcellular fractions of *E. coli* and mammalian cells.

Major Findings:

Recently work in this laboratory has been directed to studying the means by which cells regulate their levels of elongation factor Tu. By three independent means, we have shown that bacteria growing at very different growth rates contain 5 to 7 molecules of Tu per ribosome. These data, which are now being prepared for publication, are in contrast with those found by others for the elongation factors Ts and G, which are present at one copy each per ribosome. The question arises as to why does the cell require so much Tu, and efforts are now underway in an attempt to answer it. Data obtained earlier in this laboratory suggested that Tu could exist as an aggregate, and it is possible that in protein synthesis Tu functions as a polymer. It is also possible that Tu has functions in the cell other than those of protein synthesis.

We have devised a new method for the simple analysis of methylated amino acids based on autoradiography. With this technique a survey of protein methylation in a prokaryote *E. coli* and a eukaryote, fibroblasts in culture, was carried out in an attempt to identify, quantitate, and determine the subcellular localization of all the methylated amino acids found in the protein of these organisms.

In mammalian cells using an established mouse fibroblast line (3T3), we have found that nuclei-free and mitochondria-free cytoplasm contain at least four identifiable methylated amino acids: $N^{\varepsilon},N^{\varepsilon}$-dimethyllysine, $N^{\varepsilon},N^{\varepsilon},N^{\varepsilon}$-trimethyllysine, N^{G},N^{G}-dimethylarginine (or N^{G}-methylarginine), and N^{G},N'^{G}-dimethylarginine. The crude nuclear pellet also contains four identifiable methylated amino acids, but in contrast to the cytoplasmic fraction, N^{ε}-methyllysine is present and N^{G},N^{G}-dimethylarginine is absent. Qualitatively, the nuclear methylated amino acids were identical to those found in purified nuclear histones.

The ribosomal subunits of the mammalian cells contained only small amounts of the methylated amino acids; the 40S subunit contained a substantial amount of just one, N^{G},N^{G}-dimethylarginine (or N^{G}-methylarginine), and smaller amounts of N^{G},N'^{G}-dimethylarginine, and an as yet unidentified methylated amino acid. The 60S subunit contained even smaller amounts of methylated amino acids, 50% of which was $N^{\varepsilon},N^{\varepsilon},N^{\varepsilon}$-trimethyllysine and smaller amounts of N^{ε}-methyllysine, $N^{\varepsilon},N^{\varepsilon}$-dimethyllysine, and N^{G},N'^{G}-dimethylarginine.

These results were in marked contrast to those that we obtained with the prokaryote, *E. coli*. Only the proteins of the 50S ribosomal subunit of the bacteria contained methylated amino acids. Of those present 50% was $N^{\varepsilon},N^{\varepsilon},N^{\varepsilon}$-trimethyllysine, with the remainder distributed about equally between N^{ε}-methyllysine and 3 unknowns. All of the N^{ε}-methyllysine was apparently in the small acidic proteins, L7 and L12.

In addition, the autoradiographic method has allowed us to detect at least 5 new minor but yet unidentified methylated amino acids, 3 in *E. coli*, and one of these and 2 others in mouse fibroblasts.

Keyword Descriptors: protein synthesis - elongation factors - methylated amino acids

Honors and Awards: None

Publications:

Klagsbrun, M., and Furano, A. V.: Methylated amino acids in the protein of bacterial and mammalian cells. Arch. Biochem. Biophys., 1975, in press.

Project No. Z01 AM 23,630-10 LBP
1. Biochemical Pharmacology
2. Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biology of Complex Carbohydrates

Previous Serial Number: NIAMDD-LBP-4

Principal Investigator: Dr. Victor Ginsburg

Other Investigators: Drs. David A. Zopf, Kenneth C. Joseph, and Chao-Ming Tsai

Cooperating Units: None

Man Years:

Total:	4.0
Professional:	3.7
Others:	0.3

Project Description:

Major Findings:

Antibodies produced by immunization with purified oligosaccharides coupled to polylysine specifically bind complex carbohydrates containing the same nonreducing terminal sugar sequence. For example, goat antibodies against lacto-*N*-difucohexaose I (a human Le^b blood group hapten) react specifically with human $Le(a^-b^+)$ erthrocytes and with soluble Le^b blood group substance. Antibodies produced in rabbits against lacto-*N*-fucopentaose II (a human Le^a blood group hapten) exhibit similar Le^a specificity. In addition, these antibodies specifically bind the corresponding free sodium borotritide-reduced haptens.

Both antibodies can be purified by affinity chromatography on Sepharose substituted with the appropriate oligosaccharide. Affinity columns were prepared by reacting cyanogen bromide-activated Sepharose with derivatives of the oligosaccharides coupled to β(p-aminophenyl)ethylamine as previously described. Radioimmune assay systems have been developed using these antibodies and the corresponding labeled haptens. The assays can detect 0.01 nanomoles of the respective nonreducing sugar sequences in glycoproteins and glycolipids.

Since this method is 2 to 3 orders of magnitude more sensitive than currently available procedures, it is potentially useful in diagnosis of certain tumors known to accumulate Lewis blood group-active glycolipids.

In addition, the affinity purified anti-lacto-*N*-difucohexaose I antibody might be used to advantage as a routine Le(a^{-}b^{+}) erythrocyte typing reagent in clinical blood banks since human donors with high titer anti-Le^{b} isoagglutinins are rare.

Proposed Course:

Oligosaccharides of human milk will be isolated and characterized. Certain oligosaccharides will be coupled to macromolecules in order to make antibodies specific for certain sugar sequences. The antibodies will be used in radioimmune assays for the occurrence of these sugar sequences in the glycolipids and glycoproteins of cell membranes and serum.

Waldenström macroglobulins and myeloma proteins will be screened for their ability to react with human erythrocytes. Those that do will be tested for their ability to react with various oligosaccharides coupled to polylysine in order to define their specificity.

Keyword Descriptors: glycolipids - Lewis blood groups - affinity chromatography - antibodies - radioimmune assays

Honors and Awards: None

Publications:

Jeffrey, A. M. Zopf, D. A., and Ginsburg, V.: Affinity chromatography of carbohydrate-specific immunoglobulins: coupling of oligosaccharides to Sepharose. Biochem. Biophys. Res. Commun. 62: 608-613, 1975.

Zopf, D. A., and Ginsburg, V.: Carbohydrate antigens of cell surfaces. In Lee, E. Y. C. (Ed.): Biology and Chemistry of Eucaryotic Cell Surfaces. New York, Academic Press, 1974, Vol. 7, pp. 259-271.

Zopf, D. A., and Ginsburg, V.: Preparation of precipitating antigens by coupling oligosaccharides to polylysine. Arch. Biochem. Biophys. 167: 345-350, 1975.

Project No. Z01 AM 23,930-06 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of Subunit Interactions in Enzyme Chemistry

Previous Serial Number: NIAMDD-LBP-12

Principal Investigator: Dr. Claude B. Klee

Other Investigators: None

Cooperating Units: Drs. Louis A. Cohen and Kenneth L. Kirk, Laboratory of Chemistry, and Dr. Peter McPhie, Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Metabolism, and Digestive Diseases

Man Years:

Total:	0.0
Professional:	0.0
Others:	0.0

Project Description:

This project was terminated on January 19, 1974.

Publications:

Klee, C. B.: Stereospecific irreversible inhibition of histidine ammonia-lyase by L-cysteine. Biochemistry 13: 4501-4507, 1974.

Klee, C. B., Kirk, K. L., Cohen, L. A., and McPhie, P.: Histidine ammonia-lyase: the use of 4-fluorohistidine in identification of the rate-determining step. J. Biol. Chem. 250, 1975, in press.

Project No. Z01 AM 23,960-09 LBP
1. Biochemical Pharmacology
2. Biochemistry of Cell Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Cellular Activity by the Action of Pharmacodynamic Agents on Enzymes within the Cell or Its Membrane

Previous Serial Number: NIAMDD-LBP-11

Principal Investigator: Dr. Leonard D. Kohn

Other Investigators: Drs. Brian R. Mullin, Ramon L. Tate, and George Lee

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Dr. George R. Martin, Laboratory of Biochemistry, National Institute of Dental Research, NIH
Messrs. Arthur R. Schultz, Jr., John I. Powell, and Richard Shrager, Division of Computer Research and Technology, NIH

Man Years:

Total:	5.0
Professional:	4.0
Others:	1.0

Project Description:

Objectives:

The thrust of work in this section is toward an understanding of the mechanisms regulating cell activity. Special emphasis is on studies concerning the action of pharmacodynamic agents on enzymes within the cell and in its membrane. Specifically, studies have been initiated which concern the mechanism of exophthalmos and Graves' disease, preventive therapeutic measures for these diseases, and hormone receptor function at a membrane level. These studies have been extended to the enzymatic mechanisms of membrane transport in bacterial and mammalian cells; to membrane structure; to enzymatic mechanisms in the biosynthesis of collagen and their relation to human collagen disease states; and to the structure and function of oxido reductases important to energetic regulation of cell functions.

Major Findings:

I. Studies Concerning the Mechanism of Exophthalmos and of Thyrotropin Receptor Function. We have suggested that malignant exophthalmos in humans is a two-factor disease caused by the simultaneous presence of thyrotropin (TSH) or a derivative of the TSH molecule with no thyroid stimulating ability and by the presence of an abnormal or autoimmune gamma globulin. The TSH or TSH derivative served as the direct effector while the gamma globulin targeted this effector by increasing its binding to retro-orbital tissue. Our evidence which led to this hypothesis can be summarized as follows: [1] the thyrotropin molecule is exophthalmogenic; [2] the thyrotropin molecule can be fragmented by partial pepsin digestion to yield a derivative (EPF) with exophthalmogenic activity but no thyroid stimulating ability; [3] this derivative (as well as TSH) can induce exophthalmos and the biochemical changes characteristic of exophthalmos in the guinea pig, a mammalian model of exophthalmos; [4] there is an exophthalmogenic factor in the sera of exophthalmic patients which is in the gamma globulin fraction of these sera and is not the long acting thyroid stimulator (LATS); and [5] this gamma globulin from the sera of exophthalmic patients significantly increases the *in vitro* binding of TSH and EPF to retro-orbital tissue plasma membranes but not to thyroid plasma membranes.

In studies reported this year we have shown the following:

(1) The exophthalmogenic derivative (EPF) which is obtained by partial pepsin digestion of the bovine thyrotropin molecule contains two major polypeptide components with approximate molecular weights of 14,000 and 6,000. Amino acid analyses, carbohydrate analyses, and tryptic digestion experiments indicate that this exophthalmogenic factor is composed of an intact or nearly intact β subunit of thyrotropin and an amino terminal fragment of the α subunit of thyrotropin. Neither polypeptide component of the exophthalmogenic factor has the *in vivo* exophthalmogenic activity of the intact structure. *In vitro* the intact exophthalmogenic derivative of the thyrotropin molecule can bind to the thyrotropin receptor on thyroid membranes less efficiently than thyrotropin but significantly better than either its own polypeptide components or the α or β subunits of thyrotropin. The exophthalmogenic factor and its parent thyrotropin molecule can stimulate adenylate cyclase activity in retro-orbital tissue membranes from guinea pigs, a mammalian model of exophthalmos; its polypeptide components have little or no such activity.

(2) The *in vitro* binding properties of thyrotropin receptors on both retro-orbital tissue and thyroid tissue plasma membranes have been further characterized and are effectively identical in regard to pH optima, temperature effects, salt inhibition, and inhibition by other hormones, thyrotropin analogs, or thyrotropin subunits. Both receptors have a similarly heterogeneous population of receptors in regard to their affinity for thyrotropin and both have negative Hill plots suggesting a negatively cooperative relationship among the

receptors. Despite these functional similarities, gamma globulin from patients with malignant exophthalmos enhances thyrotropin binding to retro-orbital tissue plasma membranes but not to thyroid plasma membranes. Normal gamma globulin and gamma globulin from Graves' disease patients without exophthalmos do not have this effect. Tryptic digestion of thyroid plasma membranes releases a specific thyrotropin receptor 15,000 to 30,000 in molecular weight, whereas tryptic digestion of retro-orbital tissue membranes releases a 75,000 to 150,000 molecular weight thyrotropin receptor. This structural difference may correlate with the different effects of exophthalmic gamma globulin on binding in these two membrane preparations.

(3) The gamma globulin from malignant exophthalmos patients enhances TSH binding or the binding of its exophthalmogenic derivative to retro-orbital tissue plasma membranes by increasing the number of TSH receptors, by shifting the association constants of receptors to much lower levels, and by decreasing the negatively cooperative relationship amongst the receptor sites. Most important in this regard, the gamma globulin does not modify the binding site or the receptor directly. Its action requires the presence of TSH or its exophthalmogenic derivative; hence, the gamma globulin must function either to modify the TSH or EPF molecule itself or to interact with the membrane only after TSH has been bound.

(4) Retro-orbital tissue membranes have been shown to have adenylate cyclase activity which can be stimulated by throtropin and by an exophthalmogenic factor derived from the thyrotropin molecule by partial pepsin digestion. This stimulable activity is maximal after 15 minutes and is optimal in the presence of 3 mM magnesium and 1.5 mM ATP. Studies of stimulable activity as a function of hormone concentration show that thyrotropin and the exophthalmogenic factor have similar maximal levels of stimulation but that 4- to 5-fold higher concentrations of the exophthalmogenic factor are required to achieve this level. Double reciprocal plots of the hormone stimulable adenylate cyclase activity as a function of the thyrotropin or exophthalmogenic factor concentration indicate negative cooperativity or the existence of groups of TSH receptors with high and low affinity.

At 10 mM concentrations, fluoride stimulates adenylate cyclase activity 2- to 3-fold higher than either thyrotropin or the exophthalmogenic factor. Calcium salts are exquisitely inhibitory to hormonal stimulation of cyclase activity; sodium, lithium, and ammonium salts are significantly less inhibitory. Thyrotropin, luteinizing hormone, the β subunit of thyrotropin, and the α subunit of thyrotropin have relative activities for stimulation of cyclase activity of 100 : 2 : 2 : < 0.5. Several other polypeptide and glycoprotein hormones have no effect.

The gamma globulin from patients with malignant exophthalmos has no significant effect either on cyclase activity alone or on cyclase activity in the presence of maximal levels of thyrotropin or exophthalmogenic factor; this gamma globulin does, however, stimulate cyclase activity at submaximal hormone levels. Trypsin destroys the hormone stimulable adenylate cyclase activity of retro-orbital tissue plasma membranes at the same rate as it destroys hormone binding activity to these membranes. Although binding activity can be recovered as a trypsin solubilized receptor fragment, this fragment lacks both basal adenylate cyclase activity and thyrotropin stimulable activity.

(5) The thytrotopin receptor from bovine thyroid plasma membranes has been solubilized using lithium diiodosalicylate and an assay to measure thyrotropin binding to the solubilized receptor has been developed. Both the solubilized thyrotropin receptor and the thyrotropin receptor on thyroid plasma membranes have effectively identical nonlinear Scatchard plots and negatively sloped Hill plots, *i.e.*, both preparations have receptors which appear to exhibit a similar negatively cooperative relationship. Although the pH optimum of thyrotropin binding to the solubilized receptor is the same as that of the thyroid plasma membrane receptor, pH 6.0, the pH dependency curve of the solubilized receptor is slightly different in its outline. Thyrotropin binding to the solubilized receptor is less sensitive to salt inhibition than is binding to the thyroid plasma membrane receptor; however, optimal binding remains at 0°. The relative affinities of thyrotropin and two glycoprotein hormones which can be considered structural analogs, luteinizing hormone, and human chorionic gonadotropin, are 100 : 10 : 5, respectively, toward plasma membrane receptors, but 100 : 25 : 40 toward the solubilized receptors.

The solubilized receptor preparation is heterogeneous in size in that it has binding components with molecular weights of 286,000, 160,000, 75,000, and 15,000-30,000. Tryptic digestion converts all three higher molecular weight components to the 15,000 to 30,000 molecular weight species, and the 15,000 to 30,000 molecular weight receptor component has all of the binding properties of the solubilized receptor preparation before tryptic digestion including an identical nonlinear Scatchard plot. It has the same size as and co-elutes from Sephadex G100 with a 15,000 to 30,000 molecular weight receptor released by tryptic digestion of bovine thyroid plasma membranes or tryptic digestion of bovine or dog thyroid cells in culture. The tryptic fragment of the solubilized receptor preparations has been purified almost 250-fold by affinity chromatography on thyrotropin - Sepharose columns. The binding activity is lost when the solubilized thyrotropin receptor preparation is exposed to beads of neuraminidase - Sepharose or conconavalin A - Sepharose.

(6) Dog, human, and bovine thyroid cells in culture have been shown to develop follicle-like structures when cells are cultured in conditions of confluency and when cells are incubated in the presence of bovine thyrotropin or dibutyryl cyclic AMP during the first 24-48 hours after trypsinization. If thyrotropin is added 48 hours after trypsinization, these cells do not form follicle-like structures but remain as a monolayer culture. Although thyroid cells which grow as a monolayer have a thyrotropin receptor on their plasma membranes with the same *in vitro* binding properties as the thyrotropin receptor on the plasma membranes of the follicle-forming thyroid cells, there is a 1- to 2-fold greater number of receptors per mg of membrane protein when follicle-forming and monolayer cultures are compared. Follicle development under the influence of thyrotropin is associated with a 3-fold increase in cyclic AMP levels compared to cyclic AMP levels in monolayer cultures grown in the absence of thyrotropin. Despite the fact that monolayer cells have a thyrotropin receptor and despite the fact that thyrotropin can elevate cyclic AMP levels in monolayer cultures to the same level as in cultures with follicles, monolayer

cultures can no longer form follicles and have lost their functional ability to trap and organify iodide.

Non-confluent cultures of dog, bovine, and human thyroid cells react differently to thyrotropin than do confluent cultures. These cultures do not form follicles but instead grow at much faster rates than nonconfluent cultures grown in the absence of thyrotropin. The trophic effect of thyrotropin on non-confluent thyroid cell cultures appears to be analogous to the trophic effect thyrotropin has on thyroid glands *in vivo*.

Trypsinization of the cultured thyroid cells releases a 15,000 to 30,000 molecular weight receptor component into the media which specifically binds thyrotropin and can be labeled by prior incubation of the cells with [^{14}C]-glucosamine. This receptor component as well as the receptor component which is released by trypsinization of bovine thyroid plasma membranes or which can be formed by trypsinization of solubilized thyrotropin receptor preparations from bovine thyroid glands have similar properties of size and a similarly high content of carbohydrate (30%) and sialic acid (10%). Coincident with the loss of the 15,000 to 30,000 molecular weight receptor fragment into the media after trypsinization, thyrotropin binding activity on the plasma membranes of the thyroid cells is lost. When placed in fresh media, however, the cells regenerate their thyrotropin receptors as measured by the return of binding activity on their plasma membranes. Parallel with both the loss and regeneration of receptor binding activity, the thyroid cells lose and regain their ability to respond to thyrotropin as measured by the ability of thyrotropin to elevate cyclic AMP levels. The loss and regain of thyrotropin stimulable adenylate cyclase activity is not accompanied by any significant changes in basal or fluoride stimulable adenylate cyclase activity.

(7) The exophthalmogenic activity of the β subunit of bovine thyrotropin is only 10% to 20% that of the thyrotropin molecule or of an exophthalmogenic factor produced by partial pepsin digestion of purified thyrotropin preparations. The α subunit of thyrotropin, luteinizing hormone, and both subunits of luteinizing hormone have no exophthalmogenic activity.

(8) Mouse tumor thyrotropin preparations have been shown to have exophthalmogenic activity using a fish bioassay. Partial pepsin digestion of the mouse tumor thyrotropin uncovers additional exophthalmogenic activity whereas thyroid stimulating activity is rapidly destroyed; thus after 30 minutes of pepsin digestion, mouse tumor thyrotropin has 130% of its initial exophthalmogenic action but only 10% of its thyroid stimulating activity. Human thyrotropin and bovine thyrotropin behave similarly in regard to partial pepsin digestion; after 30 minutes of pepsin digestion both preparations have 80% of their exophthalmogenic activity and only 20-30% of their thyroid stimulating activity. In analogy with experimental work on bovine thyrotropin, these findings suggest that subsequent purification studies using partial pepsin digests of both purified human and purified mouse tumor thyrotropin preparations will yield an exophthalmogenic fragment of the TSH molecule devoid of any thyroid stimulating action.

(9) Clinical trials concerning the use of the MIF assay to detect those patients with hyperthyroidism who will get exophthalmos and concerning preventive therapy with Imuran have been extended in Belgium by Dr. Roger J. Winand.

Of 600 patients, about 1/3 are MIF positive and have been treated, whereas about 2/3 are MIF negative and have not been treated. In neither group has exophthalmos appeared as a complication of antithyroid therapy, despite the expectation that 25 to 100 patients would have suffered this complication in the absence of this program.

Present work is proceeding as follows: (1) Purification of a pepsin derivative of human TSH to obtain a human EPF derivative. (2) Studies of TSH biosynthesis *in vitro, i.e.*, at a polysomal level, to see if precursors of TSH or its subunits are exophthalmogenic or if degradative pathways exist to yield an EPF from such precursor material. (3) Purification of MIF in order to develop a radioimmunoassay which can replace the MIF migration assay. (4) Purification of the MIF inducing antigen starting from crude retro-orbital tissue and crude thyroid tissue extracts. (5) Characterization of the "purified" TSH receptor fragment isolated from bovine plasma membranes. (6) Studies of TSH effects on thyroglobulin synthesis and on transport in cultured thyroid cells. (7) Purification and characterization of the retro-orbital tissue TSH receptor.

II. Studies Concerning the Biosynthesis of Collagen. Antisera to dermatosparactic procollagen have been produced which are specific for the amino-terminal peptides of the procollagen molecule and do not at all react with native or heat denatured collagen. The antisera react with bovine procollagen extracted from dermatosparactic tissues or fibroblast cultures or with human procollagen extracted from tissues or fibroblast cultures but not with mouse, rat, or rabbit procollagen analogously obtained. It does not react with fetal (type III) collagen or procollagen, nor with basement membrane (type IV) collagen or procollagen. The antisera can precipitate procollagen synthesized *in vitro* on polysomes from bovine and human fibroblasts but not from mouse fibroblasts. Studies using antisera to specifically precipitate and quantitate procollagen synthesis during the growth of fibroblasts in culture show that procollagen concentrations in the media peak in early log phase, then decrease in medium and late log phase as procollagen peptidase activity increases in the media. Biosynthesis studies must exclude fetal sera in media as it and amniotic fluid have high concentrations of the amino-terminal peptides of the procollagen molecule which can interfere with radioimmunoassay studies.

In addition, a collagenous protein has been purified from bovine amniotic fluid by sequential application of ammonium sulfate fractionation, dialysis at several hydrogen ion concentrations, agarose, and DEAE chromatography. The yield is 10 mg/500 ml of amniotic fluid. It is a disulfide linked protein with a molecular weight of 410,000 to 430,000. Reduction and denaturation results in its forming a major collagenous component with a molecular weight of 144,000 and a minor one with a molecular weight of 124,000. After limited cleavage with pepsin, a single chain is obtained which has the electrophoretic mobility of an α_1 type chain. The collagenous product obtained from the pepsin digests has the amino acid and peptide composition of skin type III collagen. We conclude, therefore, that the originally purified collagenous protein is type III procollagen. An antiserum produced to this material does not react with type I collagen or procollagen, with dermatosparactic procollagen, and with type III collagen, *i.e.*, it is specific for the precursor portion of the molecule. It cross reacts with human type III procollagen. The antiserum has been used to show that abnormal amounts of type III procollagen are produced by cells from patients with connective tissue disorders, *i.e.*, osteogenesis imperfecta, and that elevated levels occur in the sera of some patients with some liver diseases, *i.e.*, acute hepatitis and vinyl chloride induced liver disease.

III. Studies Concerning Membrane Structure and Function. Recent experiments demonstrate that crude or purified preparations of D-lactate dehydrogenase (D-LDH) reconstitute D-lactate dependent oxygen consumption and active transport in membrane vesicles prepared from *E. coli* mutants (*dld*-) which are deficient in D-LDH activity. Reconstitution involves binding of D-LDH to the mutant vesicles in such a manner that electrons are transferred from the enzyme to oxygen via the electron transfer chain in the mutant vesicles. Although transport activities in the reconstituted system are similar to those obtained with wild-type vesicles and the amount of D-LDH bound relative to lac carrier protein is comparable in both systems, evidence has been presented which indicates that the D-lactate dependent transport systems have not been completely restored to their native state. In this regard, the suggestion has been made that D-LDH is bound to the outer surface of the reconstituted vesicle membrane, as opposed to the inner surface of the membrane in the native system.

Using an antiserum to D-LDH which we prepared and characterized, we examined the effects of the antibody on D-lactate dehydrogenase activity and D-lactate dependent active transport in native *E. coli* ML 308-225 membrane vesicles and ML 308-225*dld-3* vesicles reconstituted with D-lactate dehydrogenase. The results demonstrate that D-lactate dehydrogenase is inaccessible to antibody in native ML 308-225 vesicles, but readily accessible to antibody in reconstituted *dld-3* vesicles. The findings indicate that D-lactate dehydrogenase is located on the inner surface of native ML 308-225 vesicles and on the outer surface of reconstituted *dld-3* vesicles. The results with the native vesicle preparations also provide further evidence that virtually none of the vesicles is inverted or sufficiently damaged to allow access of antibody to D-lactate dehydrogenase. In addition, experiments were performed which demonstrate that an impermeable electron carrier, reduced 5-N-methyl-phenazonium-3-sulfonate, drives active transport in native ML 308-225 vesicles as well as its permeable analog reduced phenazine methosulfate. Thus, reduction of the respiratory chain from either side of the vesicle membrane is able to drive active transport.

Ca^{2+}, Mg^{2+}-Stimulated ATPase is also inaccessible to antibody in ML 308-225 vesicles unless the preparation is subjected to ultrasonic sound, incubated in Tris buffer at pH 9.0, or homogenized vigorously. As opposed to D-lactate dehydrogenase and cytochrome b_1, ATPase is readily lost from the membrane during the preparation of vesicles.

In regard to the red cell membrane, the insoluble peptide, T(is), prepared by trypsin hydrolysis of the MN-glycoprotein (glycophorin) of the human erythrocyte has been incorporated into phospholipid membranes in the form of liposomes and black lipid membranes. The permeability of liposome membranes to $^{42}K^+$ and of black lipid membranes to water and ions is increased significantly by the presence of the T(is) peptide. Electrophoresis measurements indicate that these effects are not due to the T(is) peptide carrying a net charge. The results suggest that the peptide causes local disordering of the bilayer membrane structures. The MN-glycoprotein thus appears to penetrate through the cell membrane via a nonpolar segment of its polypeptide chain, which is contained intact within the T(is) peptide. The T(is) peptide is partially helical when associated with phospholipid and forms multimeric 8.0 nm structures within the hydrophobic plane of phospholipid bilayers.

IV. Studies Concerning Structure and Function of Oxido Reductases. Oxido reductases which convert glyoxylate, hydroxypyruvate, tartrate, and malate to glycolate, D(-)-glycerate, oxaloglycolate, and oxaloacetate, respectively, have been purified to homogeneity and characterized. Experiments have been directed towards understanding their mechanism of action, the mechanism by which they achieve specificity, and the mechanism by which their subunits associate to yield active enzyme. Present experiments continue to involve sequence procedures of the active site of the induced and constitutive hydroxypyruvate reductases.

V. Studies Concerning Centrifugation. As a cooperating unit, centrifugation studies were performed for the following individuals in NIAMDD: Paul Torrence, Kenneth Ingham, Edith W. Miles, and William B. Jakoby; and for the following individuals outside NIAMDD: Thressa C. Stadtman (NHLI) and Othmar Gabriel (Georgetown University).

To improve the service aspect of this work, a direct online computer system has been developed using an in house minicomputer calculator. The minicomputer has also been used to evaluate dual label studies by personnel in Building 4, using a new and innovative procedure which minimizes errors and chance crossover. The computer is also online already for kinetic data from a Gilford spectrophotometer. This not only is one of the first uses of a low-cost minicomputer for these applications, but we believe that extension of its applications will become a significant factor in decreasing Institute and NIH-wide computer costs.

Significance to Biomedical Research and the Progress of the Institute:

The research is aimed at evaluating enzymatic mechanisms by which cellular activities are regulated by pharmacodynamic agents. The possibility that unregulated biosynthetic or degradative processes may convert TSH into EPF active material has been established, and a mechanism for human exophthalmos has been proposed on the basis of these data. A diagnostic method has been developed which predicts Graves' disease patients who will get exophthalmos during therapy and a therapeutic program to treat these patients is being tested. Our studies of type I and type III procollagen and procollagen peptidase in regard to collagen biosynthesis have become important in understanding fetal development and aging, as well as the pathogenesis of several connective tissue diseases. Diagnostic applications of our immunoassay procedures have been initiated in regard to the detection of nonafflicted carriers of several connective tissue diseases.

Proposed Course:

Studies of the TSH-EPF relationship will be continued in an attempt to examine the mechanism by which EPF activity is generated in humans *in vivo*. The proposed diagnostic mechanism for human exophthalmos will be further tested as will the therapeutic program. The mechanism of procollagen peptidase formation and control will be related to normal and disease states. The biosynthesis of type I procollagen and type III procollagen will be studied. The enzymatic mechanism of active transport in bacteria will be further defined, and studies on thyroid cell transport will be initiated. Structure function analyses of the reductase model system are being pursued. The localization of the active and regulatory sites on each enzyme will be sought and the comparative structures of these units will be compared. Comparison of membrane-bound and free enzymes will be made.

Keyword Descriptors: exophthalmos - Graves' disease - thyrotropin - exophthalmos producing factor - receptor - membrane - LATS (long acting thyroid stimulator) - adenylate cyclase - D-lactic dehydrogenase - vesicles - active transport - procollagen - procollagen peptidase - oxido reductases - minicomputers - ultracentrifugation - delayed hypersensitivity - migration inhibitory factor (MIF) - thyroglobulin

Honors and Awards: None

Publications:

Adachi, O., Kohn, L. D., and Miles, E. W.: Crystalline $\alpha_2\beta_2$ complexes of tryptophan synthetase of *Escherichia coli*. A comparison between the native complex and the reconstituted complex. J. Biol. Chem. 249: 7756-7763, 1974.

Bolonkin, D., Tate, R. L., Luber, J. H., Winand, R. J., and Kohn, L. D.: Experimental exophthalmos: binding of thyrotropin and an exophthalmogenic factor derived from thyrotropin to retro-orbital tissue plasma membranes. J. Biol. Chem. 250: August or September, 1975.

Darvey, I. G., Shrager, R., and Kohn, L. D.: Integrated steady state rate equations and the determination of individual rate constants. J. Biol. Chem. 250, in press.

Kohn, L. D., and Winand, R. J.: Characterization of the thyrotropin receptor and its involvement in exophthalmos. In Kaback, H. R., Radda, F., and Schwizer, H., Eds.: Molecular Aspects of Membrane Phenomena. Heidelberg, Germany, Springer-Verlag, in press.

Kohn, L. D., and Winand, R. J.: Experimental exophthalmos: alterations of normal hormone-receptor interactions in the pathogenesis of a disease. Israel J. Med. Sci. 10: 1348-1363, 1974.

Kohn, L. D., and Winand, R. J.: Exophthalmogenic activity of the β subunit of thyrotropin. Endocrinology, in press.

Kohn, L. D., and Winand, R. J.: Structure of an exophthalmos producing factor derived from thyrotropin by partial pepsin digestion. J. Biol. Chem. 250: August or September, 1975.

Kohn, L. D., Winand, R. J., and Bates, R. W.: Relationship of thyrotropin to exophthalmos-producing substance: formation of an exophthalmos-producing

factor by pepsin digestion of mouse pituitary tumor and human thyrotropin preparations. Endocrinology , in press.

Lea, E. J. A., Rich, G. T., and Segrest, J. P.: The effects of the membrane-penetrating polypeptide segment of the human erythrocyte MN-glycoprotein on the permeability of model lipid membranes. Biochim. Biophys. Acta 382: 41-50, 1975.

Lee, G.: Studies on procollagen and procollagen peptidase. Ph.D. Thesis, American University, December 1974.

Lichtenstein, J. R., Kohn, L. D., Martin, G. R., Byers, P., and McKusick, V. A.: Procollagen peptidase deficiency in a form of the Ehlers-Danlos syndrome. Trans. Assoc. Am. Physicians Phila. lxxxvi: 333-339, 1973.

Segrest, J. P., Gulik-Krzywicki, T., and Sardet, C.: Association of the membrane-penetrating polypeptide segment of the human erythrocyte MN-glycoprotein with phospholipid bilayers. I. Formation of freeze-etch intramembranous particles. Proc. Nat. Acad. Sci. U.S.A. 71: 3294-3298, 1974.

Short, S. A., Hawkins, T., Kohn, L. D., and Kaback, H. R.: Mechanisms of active transport in isolated bacterial membrane vesicles. XXIV. Immunochemical properties of the membrane-bound D-lactate dehydrogenase from *Escherichia coli*. J. Biol. Chem. 250, in press.

Short, S. A., Kaback, H. R., and Kohn, L. D.: Mechanisms of active transport in isolated bacterial membrane vesicles. XX. D-Lactate dehydrogenase binding in *Escherichia coli dld*⁻ membrane vesicles reconstituted for active transport. Proc. Nat. Acad. Sci. U.S.A. 71: 1461-1465, 1974.

Short, S. A., Kaback, H. R., and Kohn, L. D.: Mechanisms of active transport in isolated bacterial membrane vesicles. XXV. Localization of D-lactate dehydrogenase in native and reconstituted *Escherichia coli* membrane vesicles. J. Biol. Chem. 250, in press.

Tate, R. L., Holmes, J. M., Winand, R. J., and Kohn, L. D.: Characteristics of a solubilized thyrotropin receptor from bovine thyroid plasma membranes. J. Biol. Chem. 250: August or September, 1975.

Tate, R. L., Schwartz, H. I., Holmes, J. M., Winand, R. J., and Kohn, L. D.: Thyrotropin receptors in thyroid plasma membranes: characteristics of thyrotropin binding and solubilization of thyrotropin receptor activity by trypsin digestion. J. Biol. Chem. 250: August or September, 1975.

Tate, R. L., Winand, R. J., and Kohn, L. D.: Thyrotropin receptors and antibody. In Jakoby, W. B., and Wilchek, M. (Eds.): Methods in Enzymology. Affinity Techniques. Enzyme Purification: Part B. New York, Academic Press, 1974, Vol. 34, pp. 692-695.

Utting, J. M., and Kohn, L. D.: Structural, kinetic, and renaturation properties of an induced hydroxypyruvate reductase from *Pseudomonas acidovorans*. J. Biol. Chem. 250: in press, 1975.

Winand, R. J., and Kohn, L. D.: Pathogenesis of human and experimental exophthalmos. In Scow, R. O. (Ed.) and Ebling, F. J. G., and Henderwon, I. W. (Co-Eds.): Endocrinology: Proceedings of the Fourth International Congress of Endocrinology, Washington, D.C., June 18-24, 1972. International Congress Series No. 273. Amsterdam, The Netherlands, Excerpta Medica, 1973, pp. 1150-1155.

Winand, R. J., and Kohn, L. D.: Stimulation of adenylate cyclase activity in retro-orbital tissue membranes by thyrotropin and an exophthalmogenic factor derived from thyrotropin. J. Biol. Chem. 250: August or September, 1975.

Winand, R. J., and Kohn, L. D.: Thyrotropin effects on thyroid cells in culture: effects of trypsin on the thyrotropin receptor and on thyrotropin mediated cyclic AMP changes. J. Biol. Chem. 250: August or September, 1975.

Wolff, J., Winand, R. J., and Kohn, L. D.: The contribution of subunits of thyroid stimulating hormone to the binding and biological activity of thryotropin. Proc. Nat. Acad. Sci. U.S.A. 71: 3460-3464, 1974.

Project No. Z01 AM 24,020-12 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Chemical and Physiological Properties of the Surface of *Escherichia coli*

Previous Serial Number: NIAMDD-LBP-9

Principal Investigator: Dr. Loretta Leive

Other Investigator: Dr. William G. Coleman, Jr.

Cooperating Units: Dr. Manfred E. Bayer, Institute for Cancer Research,
Philadelphia, Pennsylvania
Dr. Shlomo Rottem, Division of Blood and Blood Products,
Bureau of Biologics, NIH

Man Years:

Total:	2.7
Professional:	2.0
Others:	0.7

Project Description:

Objectives:

To study the assembly, structure, and physiology of the outer membrane of *E. coli*.

Methods Employed:

Usual biochemical methods, including isolation and purification of cell surface components by extraction, ultracentrifugation, chromatography, and selective enzymatic degradation, determination of the size and shape of these fractions by biophysical measurements of density, viscosity, and ultracentrifugal properties, identification and quantitation of sugars by colorimetric and enzymatic procedures, use of labeled compounds to specifically label cell fractions *in vivo*, and use of immunological methods for identifying specific macromolecules. Methods for isolating and assaying bacteriophages and phage-like particles.

Major Findings:

Assembly of the Outer Membrane. Previous work from this laboratory and others indicated that there were about 10-50 specific insertion points in the outer membrane into which newly synthesized lipopolysaccharide was translocated from the inner membrane, and from whence it spread to the remainder of the outer membrane. Using a mutant which changes the antigenic specificity of its lipopolysaccharide when galactose is added to the membrane, we purified antibody specific for this lipopolysaccharide and are using it to isolate the insertion points. Cells are grown in the absence of galactose, galactose added at 0 time, and a few seconds later the cells harvested and their membranes isolated and fragmented. Membrane fragments that specifically contain newly inserted lipopolysaccharide were separated, either by precipitation with antibody specific for the new lipopolysaccharide or by fractionation on columns containing Sepharose linked to this antibody. So far we have defined the specificity of the membrane reaction with antibody and are currently working to minimize nonspecific trapping of membrane fragments which is necessary to achieve the isolation of a pure preparation of insertion points.

Structure of the Outer Membrane. The outer membrane appears by several criteria to have true membrane (lipid-protein bilayer) character, but several reports using electron spin resonance, x-ray diffraction, fluorescent probes, and other methods have suggested that it may differ in having at least some regions in which lipid mobility is restricted. It has been suggested that the lipopolysaccharide creates rigid regions by some mechanism, but there is no evidence as yet bearing on this hypothesis. Since treating cells briefly with EDTA releases half of the outer membrane lipopolysaccharide, we have begun experiments to determine whether such EDTA-treated outer membranes differ from normal outer membranes in having more regions accessible to nitroxide-labeled fatty acid probes, as analyzed by electron spin resonance.

Although EDTA treatment of *E. coli* results in release of half of the outer membrane lipopolysaccharide, electron microscopy of thin sections or negatively stained preparations revealed no differences in morphology of the membrane. However, we have now been able to demonstrate a difference in the ultrastructure of the outer face of the outer membrane after such treatment, using freeze-etch techniques. Areas of the membrane also appear thinner, and sharply differentiated from thicker "plateaus" that remain in the membrane.

Isolation and Characterization of a New Bacteriophage. *E. coli* 0111, which we have used for several years for study of the outer membrane because of its well defined lipopolysaccharide, has been reported occasionally in the literature to carry a phage-like particle. This particle was reported to kill, but not multiply in, other strains of *E. coli*. We have now analyzed this particle and have been able to make it multiply at low efficiency on other strains. We have also demonstrated that it can transduce markers from *E. coli* 0111 to other strains, indicating that it is a transducing phage, possibly defective. The relationship of this phage to other known transducing phages is currently being determined.

Significance to Biomedical Research and the Program of the Institute:

Study of the organization of the outer layer of the cell surface will contribute to understanding its structure and its interaction with the environment. Earlier work from this laboratory showed that the outer layer is the barrier to many charged molecules in coliform bacteria, including many drugs, and that this barrier can be reduced by exposing the cells to EDTA, thus making them drug-sensitive. The current studies are aimed at understanding the structural basis for this permeability barrier, and such understanding may have implications for drug therapy.

Furthermore, the very heterogeneous membrane structure suggested by the preceding experiments, in which different areas are specialized for insertion of different molecules that then can move within the membrane to achieve their final location, appears similar to the situation in cells of higher organisms, in which heterogeneity of carbohydrate-containing macromolecules on the cell surface is thought to play an important role in cell-cell interactions, differentiation, and malignancy. It is hoped that the greater ease of analyzing the outer membrane of *E. coli*, which is simpler than the membranes of higher organisms, will provide information on the mechanisms of assembling and arranging heterogeneous membranes.

Proposed Course:

Isolation and analysis of the specialized regions of lipopolysaccharide entry mentioned above will be continued. The rate of diffusion of lipopolysaccharide from these points to the rest of the outer membrane will also be measured. By specifically labeling lipid, protein, and peptidoglycan, the relationship of the insertion points for lipopolysaccharide to the location of newly inserted other components will be measured. The structure of the cell membrane before and after EDTA treatment will be studied by electron spin resonance and by accessibility to immobilized enzymes such as proteases and lipases. Such studies will help define the location of the EDTA-released moiety with respect to other outer membrane components. The nature of the above-mentioned phage will continue to be studied to determine its relation to other known coliphages.

Keyword Descriptors: outer membrane - membrane assembly - lipopolysaccharide

Publications:

Kashket, E. R., Robbins, M. L., Leive, L., and Huang, A. S.: Status of women microbiologists. Science 183: 488-494, 1974.

Leive, L.: The barrier function of the gram-negative envelope. Ann. N. Y. Acad. Sci. 235: 109-129, 1974.

Morrison, D. C., and Leive, L.: Fractions of lipopolysaccharide from *Escherichia coli* 0111:B4 prepared by two extraction procedures. J. Biol. Chem. 250: in press, 1975.

Project No. Z01 AM 24,120-04 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies in Traumatic Shock and Cellular Immunity

Previous Serial Number: NIAMDD-LBP-3

Principal Investigator: Dr. Kehl Markley

Other Investigators: Dr. Sanford M. Rosenthal (Scientist Emeritus) and
Mrs. Elizabeth T. Smallman

Cooperating Units: None

Man Years:

Total:	3.1
Professional:	2.3
Others:	0.8

Project Description:

Objectives:

I. To study the function of the lymphocyte and macrophage in traumatic shock of mice.

II. To study the influence of age and hormones on local swelling of the mouse tail and ear produced by decreased local atmospheric pressure.

Methods Employed:

I. Burn trauma was produced by immersing mice to the axilla (2/3 body surface area) in water at 70° for 7 seconds. The mice were treated with 3 ml 0.85% NaCl subcutaneously afterwards to prevent death. BALB/c mice ($H\text{-}2^d$) were immunized with EL-4 ascites tumor cells from C57CL/6N mice ($H\text{-}2^b$) injected intraperitoneally. At various time intervals afterwards, lymphocytes from the spleen, mesenteric lymph nodes, and peripheral blood were harvested and cytotoxicity of these cells was measured by determining the release of ^{51}Cr from labeled target EL-4 cells. Serum was collected from burned Swiss-Webster mice and added to the cytotoxic assay to measure its effect on cytotoxicity of splenic lymphocytes from normal, sensitized BALB/c mice.

II. Measurement of the rate of movement of fluid from the capillaries into the extravascular space was accomplished by applying a mild vacuum (-40 to -80 mm Hg) to the ear or tail of the mouse and by measuring the volume before and after the decreased atmospheric pressure. Corollary studies showed that local blood volume changes or thickening of the tissues with age had minor effects on the results.

Major Findings:

I. The effect of thermal trauma on cellular immunity was studied in inbred mice using an *in vitro* assay for cytotoxic lymphocytes from spleen, lymph nodes, and peripheral blood. BALB/c mice given a 2/3 body surface area burn in water at 70° and sensitized with tumor cells from C57BL/6N mice showed a significant decrease in cytotoxicity of spleen, lymph node, and peripheral blood lymphocytes. This depression of cytotoxicity occurred in spleen lymphocytes when the burn was given as early as 19 days before sensitization or as late as 13 days afterwards. Similar results were obtained with peripheral blood lymphocytes, but in the case of lymphocytes from lymph nodes, diminished cytotoxicity was observed only when the animals were burned after sensitization. Furthermore, serum from burned mice was able to suppress significantly the cytotoxicity of spleen lymphocytes from unburned, sensitized mice. The inhibitor in burn serum appeared to be a protein.

II. Fluid accumulation under the vacuum was shown to decrease 70% during the first 5 months of life, with a close correlation with growth rate. It is believed that this fluid movement is under hormonal control, and it has been shown that certain vasoactive hormones influence transvascular fluid movement.

Significance to Biomedical Research and the Program of the Institute:

These studies aid in the understanding of the mechanism of impaired cellular immunity after trauma and of the control and regulation of transvascular fluid movement after a vacuum.

Proposed Course:

(1) To study the function of lymphocytes and macrophages in cellular immunity after burn and tourniquet trauma. (2) To study the function of lymphocytes in gnotobiotic mice. (3) To isolate the protein in burn serum responsible for the inhibition of cytotoxicity. (4) Continue to study the effect of vasoactive hormones on transvascular fluid movement.

Keyword Descriptors: cytotoxicity - burn injury - age - transvascular fluid movement

Honors and Awards: None

Publications:

Rosenthal, S. M., and LaJohn, L. A.: Effect of age on transvascular fluid movement. Am. J. Physiol. 228: 134-140, 1975.

Project No. Z01 AM 24,140-09 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemistry and Mechanism of Pyridoxal Phosphate Enzyme

Previous Serial Number: NIAMDD-LBP-10

Principal Investigator: Dr. Edith Wilson Miles

Other Investigators: Drs. Osao Adachi and Mitsuaki Moriguchi

Cooperating Units: None

Man Years:

Total:	2.3
Professional:	2.1
Others:	0.2

Project Description:

Objectives:

To gain further understanding of features of enzymes which contribute to their conformation, subunit interaction, and mechanism of action. Pyridoxal phosphate enzymes have been chosen for study because they are important in amino acid metabolism and because pyridoxal phosphate has optical and chemical properties which make it a useful probe of the active site of the enzymes to which it is bound. The tryptophan synthase $\alpha_2\beta_2$ complex is the main enzyme under study. Since it is a multienzyme system in which one of the two protein species (the β_2 subunit) is a pyridoxal-P enzyme, it is a useful system for studying the effects of subunit interaction on the catalytic activities of the two different subunits and on the environment of amino acid residues in the active sites and interaction sites of the α and $\beta 2$ subunits.

Methods Employed:

Crystalline enzymes have been prepared and assayed by methods previously developed in this laboratory. Difference absorbance spectra and solvent perturbation difference spectra were made with a Cary 14 spectrophotometer connected to a Honeywell DDP-516 computer. Data were processed using the computer. Circular dichroism spectra were made with a Cary 60 instrument. Data were collected and processed using the computer. Fluorescence measurements were also made.

Chemical modification of tyrosyl residues with N-acetylimidazole was carried out. Modification with a substrate analog, α-cyanoglycine, has also been studied as has modification of bound pyridoxal-P with hydroxylamine or phenylhydrazine. The course of modification reactions has been followed by absorbance and circular dichroism spectra and by assays of activity.

Major Findings:

Changes in the environment of aromatic amino acids which result from the association of the α and apo β_2 complex has been evaluated by difference absorbance spectra, circular dichroism spectra, and by chemical modification with N-acetylimidazole. The association of 2 α subunits with 1 apo β_2 subunit results in the transfer of 6 to 7 tyrosine residues from an exposed environment into a hydrophobic environment. Subunit association is also accompanied by very large changes in the circular dichroism spectrum in the aromatic region which appear to be due to changes in the environment of tyrosine residues and not in that of tryptophan residues.

Reactions of the β_2 subunit and $\alpha_2\beta_2$ complex with substrate analogs which may also be irreversible inhibitors are being compared. α-Cyanoglycine inhibits both the β_2 subunit and the $\alpha_2\beta_2$ complex. Our finding that the chromophoric, α-cyanoglycine derivative of the $\alpha_2\beta_2$ complex is much more stable than the corresponding derivative of the β_2 subunit indicates that the environment of the derivative is quite different in the $\alpha_2\beta_2$ complex.

Similar conclusions are being reached from comparative studies of derivatives of pyridoxal-P bound by the β_2 subunit and $\alpha_2\beta_2$ complex. Oxime and phenylhydrazone derivatives of the holo $\alpha_2\beta_2$ complex are tightly bound, whereas the corresponding derivatives of the holo β_2 subunit are readily removed. Comparative circular dichroism studies of derivatives of the β_2 subunit and $\alpha_2\beta_2$ complex are in progress.

Keyword Descriptors: pyridoxal-phosphate - tryptophan synthase - multienzyme system - subunit interaction - difference spectra - chemical modification - aromatic amino acids

Honors and Awards: None

Publications:

Adachi, O., Kohn, L. D., and Miles, E. W.: Crystalline $\alpha_2\beta_2$ complexes of tryptophan synthetase of *Escherichia coli*. A comparison between the native complex and the reconstituted complex. J. Biol. Chem. 249: 7756-7763, 1974.

Miles, E. W.: Effects of modification of the β_2 subunit and of the $\alpha_2\beta_2$ complex of tryptophan synthase by α-cyanoglycine, a substrate analog. Biochem. Biophys. Res. Commun., in press, 1975.

Project No. Z01 AM 24,260-09 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Enzymatic Studies of Nucleic Acid Metabolism

Previous Serial Number: NIAMDD-LBP-6

Principal Investigator: Dr. Nancy G. Nossal

Other Investigators: Dr. Frances D. Gillin (NIH Special Postdoctoral Fellow) and Dr. E. Bruce Konrad

Cooperating Units: None

Man Years:

Total:	2.8
Professional:	2.5
Others:	0.3

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Major Findings and Proposed Course:

I. Proteins Required for T4 Bacteriophage DNA Synthesis

We are using *Escherichia coli* infected with T4 bacteriophage as a system in which to identify and characterize the reactions catalyzed by enzymes required for DNA replication, since this phage appears to induce the synthesis of the enzymes required for its own DNA synthesis, rather than depending on host proteins. Phage with mutations in genes coding for many of these enzymes are available due to the work of Edgar, Epstein, and their collaborators, and phage infected bacteria can be grown in quantities allowing purification of sufficient enzyme for biochemical and physical studies. The work has been facilitated by a regulatory phage mutant characterized by Wiberg which produces high levels of several of these enzymes.

Genetic studies by Edgar and Epstein *et al.* have shown that the proteins encoded by the phage genes 32 (DNA unwinding protein), 41, 43 (DNA polymerase), 44, 45, and 62 are required for DNA synthesis. Many other phage proteins are required for the synthesis of DNA precursors, or are involved in controlling the timing or extent of viral DNA synthesis. We are in the process of developing procedures for the identification and purification of these proteins.

The DNA polymerase and DNA unwinding proteins have been purified to apparent homogeneity and freed of contaminating nucleases beginning with affinity chromatography on DNA cellulose. We have shown previously that the polymerase, which has both polymerase and 3' to 5' exonuclease activities in a single polypeptide of 110,000 molecular weight, copies single stranded DNA using the 3' OH end of the chain as a primer, but cannot by itself copy linear duplex DNA's like T4 DNA. The DNA unwinding protein (35,000 molecular weight) has been shown by Alberts and his collaborators to promote DNA strand unwinding *in vitro* and to stimulate the copying of single stranded templates by T4 DNA polymerase.

We have developed a complementation assay for the other proteins required for T4 DNA synthesis *in vivo* (genes 41, 44, 45, and 62) which is based on the ability of these proteins to stimulate DNA synthesis using an endogenous template in a crude lysate of cells infected with phage defective in that protein. The gene 44 protein has been purified to apparent homogeneity. The protein purified using gene 44 complementation as an assay is actually a complex of the gene 44 and 62 proteins; gene 42 complementation activity and a DNA dependent ATPase (and dATPase) follow the gene 44 complementation activity during the purification, in agreement with the results of Alberts and coworkers for 44-62 protein purified by a different procedure. The complex does not have DNA polymerase, DNA exo- or endonuclease, or RNase H (hydrolysis of the RNA strand in an RNA · DNA hybrid duplex) activity.

The gene 45 protein has also been purified to apparent homogeneity with a high yield using the complementation assay. This protein does not bind to DNA cellulose. The purest enzyme is free of DNA polymerase, DNA nuclease, DNA dependent or independent ATPase, and RNase H activities. The 45 protein is of special interest because, in addition to its role in DNA replication, it is also required for late mRNA transcription (Geiduschek) and binds specifically to the T4 phage modified form of the host RNA polymerase (Ratner). The copying of single stranded DNA by the T4 DNA polymerase is strongly stimulated by the addition of both the gene 44-62 and gene 45 proteins.

The T4 DNA polymerase, like DNA directed DNA polymerases from other sources, is unable to initiate the synthesis of new chains. We have shown that the enzyme is able to bind to places in a duplex DNA strand with breaks in one of the strands (nicks) and to the ends of strands in a duplex. However, the polymerase by itself is unable to copy nicked duplex DNA, apparently because it is unable to displace the DNA strand which is annealed to the DNA strand serving as the template. We have also shown previously that under conditions of low salt and low Mg^{2+}, where the helix is less stable, the addition of the gene 32 DNA unwinding protein, which would facilitate strand displacement, permits limited copying of nicked duplex DNA by the polymerase. The principal products of this reaction are rapidly renaturable, branched molecules and poly[d(A-T)] which results from repetitive copying of the A-T rich region of the DNA. At higher salt and Mg^{2+} concentrations, the polymerase and unwinding protein are not sufficient for copying duplex DNA templates. Under these conditions we now find substantial synthesis if the gene 45 and gene 44-62 proteins are also present. The rate of this reaction increases with increasing concentration of each of the 4 proteins and continues at a linear rate for at least 30 minutes. The product of this reaction has been shown not to be rapidly renaturable by CsCl density gradient centrifugation following strand separation in the presence of poly rUG.

During the next year we plan to optimize conditions for the synthesis with duplex DNA and to characterize the product using enzymatic, centrifugal, and electron microscopic techniques. We hope to improve the assay for the 41 protein, which has proved less reliable than those for the other proteins, to continue the purification of this protein, and to test its ability to stimulate DNA synthesis in the presence of the other proteins with duplex and circular single stranded DNA templates. We will try to determine the reactions carried out by these proteins and the mechanism by which they stimulate the polymerase. (. . . . . Dr. N. G. Nossal)

II. Control of Mutation Rates by the T4 DNA Polymerase

T4 DNA polymerase has unique features which make it an excellent enzyme for the study of factors contributing to the fidelity of DNA replication. Temperature sensitive phage with single point mutations in the structural gene for the polymerase have been identified by Speyer, Drake, and their collaborators, which at permissive temperatures have either a markedly increased (mutator phage) or decreased (antimutator phage) frequency of mutations of markers in other genes, compared with the wild-type phage.

Since the enzyme has both polymerase and 3' to 5' exonuclease activities, changes in the accuracy of DNA replication with the mutant enzymes could result from differences in the ability of the enzymes to discriminate between correct and incorrect nucleotides during incorporation and/or from differences in the efficiency of removal of noncomplementary nucleotides by the exonuclease activities of the mutant enzymes.

We have compared the purified DNA polymerase induced by the wild-type T4 phage with that from a strong mutator (*ts* L88) and a strong antimutator (*ts* CB120). We have previously shown that with the homopolymer pair, poly dA plus poly dT, as template-primer, the mutator enzyme catalyzes the utilization of noncomplementary nucleotides (dGTP and dCTP) more frequently than the wild-type enzyme, while the antimutator enzyme utilizes each of the wrong nucleotides less frequently. With all 3 enzymes the utilization of noncomplementary nucleotides was measured by the DNA dependent conversion of deoxynucleoside triphosphate to monophosphate. There is no stable incorporation of incorrect nucleotides at the specific radioactivities used, apparently because these residues are rapidly removed by the exonuclease activity of each enzyme. These results suggested that the enzymes differed in the frequencies with which they incorporated noncomplementary nucleotides, in addition to any differences in efficiency of "editing" by nuclease removal of incorporation errors. The interpretation that the rate of utilization of noncomplementary nucleotides is not limited by the rate at which they are hydrolyzed is supported by recent studies with poly dA molecules to which a labeled dA or dG residue was added at the 3' OH chain end with deoxynucleotidyl terminal transferase. When these molecules are mixed with poly dT, the rate of removal of the terminal dA or dG by the wild-type and both mutant enzymes is much greater than the rate of DNA dependent formation of deoxynucleoside monophosphate.

Using this homopolymer pair, we have now also shown that the wild-type enzyme has a markedly higher apparent K_m for the utilization of noncomplementary nucleotides (190 μM for dGTP and 1,600 μM for dCTP) compared with those for complementary nucleotides (17 μM for dATP and 6 μM for dTTP). The apparent K_m's for the 4 dNTP are similar with a template containing the 4 bases. The mutator enzyme has K_m's for both complementary and noncomplementary nucleotides which are similar to those of the wild-type, but it has a very much higher apparent K_m than the wild-type for this and other DNA templates. The antimutator enzyme had apparent K_m's for the nucleotides which were similar to those of the wild-type and its "K_m" for polymer was also like that of the wild-type enzyme. The mutant enzymes showed interesting differences when their apparent K_m's for polymer were compared using a fixed concentration of either complementary or noncomplementary nucleoside triphosphate. The wild-type "K_m" for poly dA · poly dT was the same with dATP or dGTP. The mutator enzyme had a higher "K_m" for polymer with dATP than dGTP, while the antimutator enzymes had a higher "K_m" for polymer with dGTP than dATP.

The antimutator polymerase hydrolyzes a much larger fraction of newly incorporated nucleotides than the wild-type enzyme. Previous studies showed that the antimutator enzyme was less able than the wild-type to copy templates with intrastrand bonding, and that its synthesis with such templates was stimulated to a greater extent than the wild-type by the addition of the gene 32 DNA unwinding protein. Thus the high rate of product hydrolysis appeared to result from repetitive incorporation and removal of nucleotides at places where the enzyme is unable to go forward to complete the copying of the template. This interpretation is supported by recent experiments which show that the antimutator hydrolyzes newly incorporated nucleotides 7 to 10 times faster than the wild-type at the ends of single stranded T7 DNA molecules which can act as the primer for copying other parts of the chain but has only a 1.4-fold increase over wild-type at the ends of duplex T7 DNA molecules which neither enzyme can use as a primer for net synthesis.

Mutation rates for T4 phage have been shown by others to increase markedly during growth in Mn^{2+} *in vivo*. We have now shown that the utilization of noncomplementary nucleotides with poly dA · poly dT is increased when Mn^{2+} replaces Mg^{2+}. In addition, the wild-type enzyme utilizes measurable incorrect nucleotides with the alternating polymer, poly[d(A-T)], as the template-primer in the presence of Mn^{2+} but not with Mg^{2+} at specific radioactivities like those used for the poly dA · poly dT experiments. The addition of dATP decreases the incorporation of dGTP but increases that of dCTP, while dTTP decreases dGTP and increases dCTP. Thus the incorrect purine apparently replaces the correct purine and the incorrect pyrimidine replaces the correct one. In this system the mutator enzymes incorporate both noncomplementary nucleotides at rates which are similar to that of the wild-type enzyme and very much greater than those of the antimutator polymerase.

During the next year we would like to determine if the mutant enzymes differ in the extent to which the polymerization reaction is processive, by comparing the size of the product with time using acrylamide gel electrophoresis. The wild-type exonuclease degrades short oligonucleotides at a faster rate than long ones, perhaps because it binds nonproductivity to internal nucleotides in the long chains. We plan to pursue some preliminary studies which suggest this may occur to a much lesser extent with the mutator enzyme. We will compare the ability of the 3 enzymes to copy correctly templates containing base analogs like bromouracil. We will investigate the interaction of the gene 44-62 and gene 45 proteins with the mutant polymerases. (. . . . Dr. F. D. Gillin)

III. Isolation of a Novel Class of DNA Replication Mutants in E. coli

An exhaustive search for *E. coli* mutations which lessen the fidelity of DNA replication ("mutator" mutations) and are conditionally lethal (can grow at 30° but not 44°) has been initiated. Since DNA replication is an essential function, one would anticipate such mutations. They have been found in *Bacillus subtilis* and also in phage T4, but not in *E. coli*. This may be simply due to an insufficiently thorough search, since previously used screening procedures for isolating mutator mutations are cumbersome and limited to small numbers of clones.

To overcome this difficulty we have developed the following method for screening large numbers (up to several million) clones. Strains carry lac^- point mutations are mutagenized and plated on lactose tetrazolium indicator plates. On those plates, lac^- colonies are red; however, lac^+ mutants which arise within a lac^- colony appear as white papillae on the colony's surface. The number of papillae is roughly proportional to the frequency of lac^+ mutants. Thus colonies with a high mutation rate can be identified. Conventional methods can then be used to confirm that a mutator mutation is present and to determine whether it is conditionally lethal.

The potential usefulness of this novel class of mutator mutations can be seen from a brief consideration of the present state of DNA replication studies in *E. coli*, the prokaryotic organism where knowledge of this process is most advanced. A number of the proteins that take part in this process have been isolated. Certainly more remain to be identified and purified. The role each particular protein plays, and how it interacts with the others, is only partially understood for some areas, and unknown for most. Current studies of these problems depend heavily on *in vitro* systems consisting to various extents of cell-free *E. coli* extracts and purified proteins and capable of more or less replicating a particular chromosome, usually viral.

These *in vitro* systems in turn rely on conditionally lethal DNA replication mutants. Such mutants allow purification of replication proteins by complementation assays, and they provide tests for whether *in vitro* reactions correspond to *in vivo* ones. The physiology of such mutants can make suggestions about the function of the mutant proteins. Conditionally lethal DNA replication mutants currently available are limited to ones which block replication at the nonpermissive temperature or which block joining of replicative fragments. Conditionally lethal mutator mutants may lead to the isolation of new components of the replication machinery. They can also be expected to help identify the components which contribute to the fidelity of this process.

If we succeed in isolating conditionally lethal mutator mutations, we will follow up the work by examining the mutants' physiology and the behavior of mutant extracts in *in vitro* DNA replication systems. (. . . . . Dr. E. B. Konrad)

Significance to Biomedical Research and the Program of the Institute:

Knowledge of enzymes controlling the extent and fidelity of DNA replication is important to an understanding of the control of DNA replication and transcription in both normal and viral infected cells.

Keyword Descriptors: *E. coli* - T4 bacteriophage - DNA synthesis - DNA polymerase - DNA unwinding protein - gene 44 - gene 45 - mutator - antimutator

Honors and Awards: None

Publications:

Gillin, F. D., and Nossal, N. G.: T4 DNA polymerase has a lower apparent K_m for deoxynucleoside triphosphates complementary rather than noncomplementary to the template. Biochem. Biophys. Res. Commun., 1975, in press.

Nossal, N. G.: DNA polymerase from wild-type and mutant T4 bacteriophage. In Wickner, R. B. (Ed.): DNA Replication. Methods in Molecular Biology. New York, Marcel Dekker, Inc., 1974, Vol. 7, pp. 239-255.

Nossal, N. G.: DNA synthesis on a double-stranded DNA template by the T4 bacteriophage DNA polymerase and the T4 gene 32 DNA unwinding protein. J. Biol. Chem. 249: 5568-5676, 1974.

Project No. Z01 AM 24,710-25 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Estimation, Metabolism, and Function of Amines

Previous Serial Number: NIAMDD-LBP-5

Principal Investigators: Drs. Herbert Tabor and Celia White Tabor

Other Investigator: Dr. Edmund W. Hafner

Cooperating Units: None

Man Years:

Total:	4.5
Professional:	3.0
Others:	1.5

Project Description:

Glutathionylspermidine accumulates in *Escherichia coli* at the end of growth, and accounts for all of the intracellular spermidine and one-half of the intracellular glutathione. Added [^{35}S]glutathione or [^{3}H]spermidine are incorporated into glutathionylspermidine. Glutathionylspermidine has been isolated from *E. coli* and characterized by chemical and enzymatic techniques. Turnover studies have shown that, even when it is accumulating, glutathionylspermidine is in equilibrium with unbound glutathione and with free spermidine. Enzymes are present in *E. coli* for both the synthesis and the degradation of glutathionylspermidine.

Closely related to these studies are our studies showing that glutathione is normally present in large amounts in *E. coli*. For this purpose, a new analytical technique was developed for the automated amino acid analyzer, based on elution from an anion exchange resin. This technique permits the quantitative determination of free and total glutathione, and of related compounds, in liver and other tissues, as well as in *E. coli*.

The synthesis of adenosylmethionine is the first step in the biosynthesis of spermidine. The enzyme, which carries out this reaction in the presence of ATP, K^+, Mg^{2+}, and L-methionine, has been purified from *E. coli*. Mutants with low levels of this enzyme have been isolated by taking advantage of their resistance to ethionine, and have been shown to map at the met K locus. A mutant, which maps in the met K locus, and which is only ethionine resistant at elevated temperatures, was isolated, and shown to contain a temperature-sensitive adenosylmethionine synthetase, indicating that this is the structural gene for the enzyme.

Keyword Descriptors: spermine - spermidine - glutathione - glutathionylspermidine - methionine - adenosylmethionine - ethionine

Honors and Awards: None

Publications:

Tabor, H., and Tabor, C. W.: Isolation, characterization, and turnover of glutathionylspermidine from *Escherichia coli*. J. Biol. Chem. 250: 2648-2654, 1975.

Project No. Z01 AM 24,940-02 LBP
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanisms of Inheritance in *Saccharomyces cerevisiae*

Previous Serial Number: NIAMDD-LBP-13

Principal Investigator: Dr. Reed B. Wickner

Other Investigator: Dr. Michael J. Leibowitz, Guest Worker, LBP (Research Associate in Pharmacology, NIGMS)

Cooperating Units: None

Man Years:

Total:	2.3
Professional:	2.0
Others:	0.3

Project Description:

Objectives:

The long-term objectives are: (*I*) to understand the mechanisms of control of inheritance and expression of the killer plasmid and (*II*) to understand the mechanisms whereby chromosomal inheritance is controlled.

Methods Employed:

Standard biochemical and genetic techniques have been used.

Major Findings:

I. The Killer Character of Yeast.

(A) Background. Certain strains of *S. cerevisiae* (called killers) secrete a substance which is lethal to other strains of the same species, but has no effect on the secreting strain. The toxic material is present in the medium, is macromolecular, and does not propagate itself (form plaques). The structure of the toxin and its mechanism of action have not been well characterized.

Crosses of killer strains and nonkiller strains have revealed a cytoplasmic pattern of inheritance. The cytoplasmic killer genome is apparently a species of double-stranded RNA of molecular weight 1.5×10^6. This double-stranded RNA is encapsulated within small virus-like particles in the cell.

(B) Genetic Studies of the Killer Phenomenon. We have isolated from a killer strain a large series of mutants which have lost the ability to kill. Some of these mutants have acquired a defect in the cytoplasmic killer genome. Others have defects in chromosomal genes (*pets*, *mak1*, *mak2*, and *mak3*) needed to replicate or maintain the cytoplasmic genomes. A third group are defective in chromosomal genes (*kex1* and *kex2*) needed for killer expression, but not needed for plasmid replication or maintenance.

We have located *mak1* near the centromere of chromosome XV while *mak3* is near *tyr7* on chromosome XVI. *Kex2* is on chromosome XIV, not linked to any previously known markers on this chromosome. *Pets* is between the centromere and mating type locus on chromosome III.

We have noted that defects in some of these chromosomal genes involved in the killer phenomenon are accompanied by defects in other aspects of the life and reproduction of the cell.

1. Eleven independently isolated mutants assigned to *kex2* by complementation show reduced sporulation ability (> 200-fold) in homozygous diploids. *Kex2* haploids also show an α-specific mating defect, *i.e.*, *α kex2* strains mate with *a* strains at a very low frequency, but *a kex2* strains mate with *α* strains normally. Normal *α* strains secrete an oligopeptide, "*α* mating hormone," which prepares normal *a* cells for mating. *Kex2* strains of the *α* mating type which fail to mate, fail to secrete this hormone, but *a kex2* strains, which mate normally, respond normally to the hormone produced by normal *α* strains. The hormone defect in *α kex2* cells, while sufficient to account for their inability to mate, is not the only defect, since supplying active "*α* hormone" does not increase their mating ability. Thus, mutation of a single chromosomal gene, *kex2*, results in 3 defects: inability of *α* cells to mate, inability of homozygous diploids to undergo meiosis and sporulation, and a defect in expression of killing.

2. The *pets* gene is needed to maintain and replicate both the killer plasmid and another cytoplasmic genetic element, mitochondrial DNA. We have recently shown that this gene is also needed for normal mitotic growth, since 2 independent mutations in this gene result in temperature sensitivity for growth which co-segregated with the other defects, which are expressed even at the permissive temperature for growth.

3. Mutation of the *mak1* gene can also result in temperature sensitivity for growth, with the defect in killer plasmid replication again seen at the permissive temperature for growth. Temperature-resistant revertants have simultaneously regained the ability to maintain the killer plasmid.

(C) *Sek* Mutants. Mutants in at least 2 new chromosomal genes have been isolated which are needed for sensitivity to the killer toxin. The mutant phenotype (resistance) is recessive to the wild-type phenotype (sensitivity). Such mutants are easily isolated as the main class of survivors of a sensitive strain after co-cultivation with a killer strain.

(D) Plasmid Mutants Dependent on Host Diploidy. Mutants of the killer plasmid have been isolated which have become dependent upon chromosomal diploidy for the expression of plasmid functions and for replication or maintenance of the plasmid itself. These mutants show no evidence of defects in any chromosomal gene needed for expression or replication of the killer plasmid.

Haploids carrying these mutant plasmids (called d for diploid-dependent) may be unable to kill or unable to resist being killed or both and show frequent loss of the plasmid. The wild-type phenotype is restored by mating the d plasmid-carrying strain with either (a) a wild-type sensitive strain which apparently has no killer plasmid; (b) a strain which has been cured of the killer plasmid by growth at elevated temperature; (c) a strain which has been cured of the plasmid by growth in the presence of cycloheximide; (d) a strain which has lost the plasmid because it carries a mutation in a chromosomal mak gene; or (e) another strain which carries the same d plasmid and has the same defective phenotype.

The "selfing" experiment (e) above, along with other data, indicates that the restoration of the normal phenotype is not due to recombination between plasmid genomes or complementation of plasmid or chromosomal genes.

Sporulation of the K^+R^+ diploids formed in d X wild-type nonkiller crosses yields tetrads, all 4 of whose spores are defective for killing or resistance or maintenance of the plasmid or a combination of these. The pattern of defectiveness depends on the particular d strain used and the particular cross. These defective segregants resume the normal killer phenotype in the diploids formed when a second round of mating is performed.

II. Ribonuclease H of S. cerevisiae.

Yeast has an RNase which is specific for the RNA of DNA-RNA hybrids (RNase H). Such enzymes have been postulated to have roles in DNA replication, transcription, RNA processing, and in reverse transcription.

We have found that this enzyme can be easily and specifically assayed in crude extracts without interference from other RNases or DNases. Methods have been developed for mass screening of clones of mutagenized cells to find a mutant deficient in these enzymes.

The enzyme has also been purified several hundred fold. It seems to purify as a single enzyme. Thus, it may be possible to obtain a mutant with extracts either completely deficient in RNase H or having only abnormally thermolabile enzyme.

Our plan is to study such mutants genetically and biochemically in order to understand the role of this enzyme in the cell.

Significance to Biomedical Research and the Program of the Institute:

The killer plasmid appears to be composed of 2 species of double-stranded RNA and is associated with virus-like particles. While double-stranded RNA viruses are ubiquitous, infecting bacteria, fungi, plants, insects, and mammals (including at least 2 pathogens infecting man), this is the first example of a plasmid with a double-stranded RNA genome. The distinction may, however, be purely formal. This system provides a simple means of investigating host-virus genetic relationships as well as the mechanisms of replication, segregation, recombination, and expression of such plasmids.

Keyword Descriptors: double-stranded RNA - plasmid - virus-like particles - chromosomal genes - pleiotropic mutants - mapping - RNase H - killer *S. cerevisiae*

Honors and Awards: None

Publications:

Wickner, R. B.: Chromosomal and nonchromosomal mutations affecting the "killer character" of *Saccharomyces cerevisiae*. Genetics 76: 423-432, 1974.

Wickner, R. B.: Chromosomal mutations affecting the ability of *Saccharomyces cerevisiae* to harbor or express the "killer" extrachromosomal genome. In Robinson, W. S., and Fox, C. F. (Eds.): Mechanisms of Virus Disease. Menlo Park, California, W. A. Benjamin, Inc., 1974, pp. 421-426.

Wickner, R. B. (Editor): DNA Replication. Methods in Molecular Biology. New York, Marcel Dekker, Inc., 1974, Vol. 7, 299 pp.

Wickner, R. B.: DNA replication in plasmolyzed *Escherichia coli*. In Wickner, R. B. (Ed.): DNA Replication. Methods in Molecular Biology. New York, Marcel Dekker, Inc., 1974, Vol. 7, pp. 181-186.

Wickner, R. B.: Mutants of *Saccharomyces cerevisiae* that incorporate dTMP into DNA *in vivo*. In Prescott, D. (Ed.): Methods in Cell Biology. New York, Academic Press, 1974, Vol. 9, in press.

ANNUAL REPORT SUMMARY

LABORATORY OF CHEMICAL PHYSICS

One major area of research in this laboratory is the elucidation of detailed molecular and electronic structure and the attempt to relate that structure to the function of certain biological systems. Electron paramagnetic resonance is a very sensitive tool for studying the electron distribution at an active molecular site if a paramagnetic ion is located at the site. For example, in cytochrome P450 the Fe(III) at the active site shows extraordinarily large rhombic electronic distortion, which has been widely believed to be related to an -SH ligand attached to the iron. However, Kon and Sato, using EPR, have studied a model system for the active site of this enzyme, tetraphenylporphine Fe(III)X, where X is an anion, and have found that when surrounded by molecules of tetraphenylporphine itself in a mixed crystal, the same kind of electronic distortion is produced, thereby demonstrating that the -SH ligand in the enzyme is not a necessary condition for the unusual distortion. In one of a number of other collaborative studies, Kon and Nebert (CH-LBS) have found the EPR is sensitive to substrate-produced shifts between two spin states of cytochrome P450 which the usual method of optical difference spectroscopy is incapable of detecting. Eaton, Hanson and Hofrichter have examined cytochrome P450 by another elegant optical method, polarized absorption spectra of single crystals, and in addition, Hanson has carried out quantum mechanical calculations on the possible coordination structures deduced from the optical spectra. The results thus far indicate that the mercaptan sulfur is one of the axial ligands and although shown not necessary for the strong rhombic distortion in the model tetraphenylphorphine studied by Kon and Sato, may in fact explain the distortion in the enzyme itself.

The nuclear and electronic structure of macromolecules is being probed in another way by Charney, who in a continuing study of the behavior of DNA and polynucleotides by electric dichroism and birefringence has demonstrated that, in the absence of Mg^{++}, glycerol, which is supposed to mimic the effect of cyclic AMP in DNA transcription in λ phage by causing some structural change in the DNA, does not in fact affect the helical structure to the extent of causing a tilt of the bases. It does, however, affect the rotational relaxation of the DNA molecule in a way not expected from the known dependence of the relaxation on the viscosity of the solvent. Thus some as yet unexplained structural or solvation effect does occur.

One of the most biologically and medically significant findings this year in LCP also came as a result of spectroscopic studies of macromolecular structure and behavior, in this case of hemoglobin-S, the sickling agent in sickle cell disease. Following up earlier findings on the structure of the hemoglobin-S polymer in sickled cells, Hofrichter and Eaton, with Ross (LMB-NIAMDD) used highly sensitive spectroscopic and microcalorimetric techniques to study the formation of the gel caused by the polymerization of the hemoglobin-S. In this first kinetic study of this process, they found a most unusual time course for the gelation reaction. Hofrichter's theoretical model for the kinetics explains the time dependence in terms of approximately a 30th power dependence on the hemoglobin-S concentration. This result, combined with a striking temperature dependence, indicates that in vivo gelation, the cause of the sickling process, takes place in times comparable to capillary transit and venous circulation times, a fact which may have profound significance in sickle cell disease therapy.

A number of spectroscopic studies of smaller molecules have been completed or are underway. Trans-1,3-butadiene is the simplest polyene and thus the forerunner of unsaturated systems. McDiarmid has examined the spectrum of the molecule at high vibronic resolution down to 1350 Å. A previously unobserved electronic transition at slightly higher energies than the main strong transition of the π electron system at 2150 Å has been found and analyzed. Several of the absorption systems previously observed at higher energies by other investigators have been found to have been misassigned. The new assignments will be extremely helpful in describing the electronic structure of the conjugated π electron systems (which occur in so many biologically important organisms). Using quantum theoretical methods, Charney and Rosenfield have analyzed several of the higher electronic states of the diketone system in camphorquinone, a molecule whose lower states were studied earlier by Charney and Tsai. Rosenfield has also used quantum calculations to show that a feature in the magnetic circular dichroism spectrum of benzene can be attributed to a state not previously expected to be in the region in which it is found.

Levin and co-workers have studied the gas phase Raman spectrum of PF_5 and used a two-dimensional harmonic oscillator to explain the axial-equatorial fluorine exchange resulting from pseudorotation in this molecule. Pearce and Levin developed a modified internal valence force field to account for the in-plane vibrational modes of 1-methyluracil and several isotopic derivatives, and, with Spiker, they accounted for vibrational interactions in dimethyl ether in terms of methyl-methyl interactions. Levin and Spiker used Raman spectroscopy to probe the structure of phospholipid-water bilayer vesicles and to monitor vibrational motions that are dependent on molecular conformation.

Pinnavaia examined the proton NMR spectra of guanosine-5'-phosphate (GMP) under conditions where Miles (LMB-NIAMDD) has shown by infrared spectroscopy that specific molecular interactions occur. Surprisingly slow exchange processes were found to occur between the monomeric and interacted forms, thus permitting the direct observation of the NMR spectrum of the interacted form, for which a tentative octamer structure has been proposed. Rao and Becker also employed NMR to examine the interaction of metal ions, such as Li^+, with simple amides and to elucidate the effect of the interaction on barriers to internal rotation of methyl groups within the amides.

Ziffer and Kabuto have embarked on an investigation of the synthesis of optically active organic compounds in reactions favoring a particular enantiomer. They have been able to synthesize cis and trans-3-phenyl-1,2-cyclohexane diols in 25-40% enantiomeric excess. They have developed the use of optically active NMR shift reagents as a method of analyzing the enantiomeric mixture. Ziffer and co-workers have also continued their studies of the photochemistry of β,γ-unsaturated ketones by establishing the triplet energy in one reaction, measuring the triplet lifetime of another such ketone, and demonstrating in a third example that photoepimerization changes the structure from a more stable (ground state) to a less stable one.

Yoshihira and Weiss are attempting to elucidate the structure of the antibiotic chrysomycin, which has been found actually to consist of two related substances, chrysomycin-A and -B. Weiss and Lousberg have synthesized 1-fluorocodeine, which has been found by May (LC-NIAMDD) to have analgesic action close to that of codeine itself. Thus the long-known diminished activity of the corresponding chloro- and bromocodeines must be due to the bulk of the substituent, not to electronic effects.

Shean has verified experimentally the earlier theoretical work by Sollner showing that Donnan membrane equilibria permit simultaneous accumulation of both cations and amions. For example, across an appropriate mosaic membrane a spontaneous concentration enhancement of a factor of 30 was observed for sodium acetate when the corresponding KCl concentration ratio was maintained at 100.

Adams and Sharpless have continued their studies of possible models for the chemical structure of rhodopsin, the light-absorbing pigment of vertebrate night vision. Attempts have been made to synthesize a tri-peptide, lysyl-alanyl-phenylalanine that closely resembles the amino acid sequence of the peptide chain of vertebrate rhodopsin near the site of attachment of its carotenoid chromophore. The hypothesis is proposed that rhodopsin's peculiar spectral properties are due to a) Schiff base formation between retinaldehyde and the lysyl ε amino group, and b) a π-π electronic interaction between the conjugated electron chain of retinal and the aromatic ring of phenylalanine.

Yoshikami, Robinson and Hagins have studied the ionic metabolism of retinal rod and cone cells by analysis of quick frozen live retinas with an electron microprobe. The ionic compositions obtained support previous conclusions that the cells have low resting potentials due to the presence of a large "dark current" resulting from entry of Na^+ ions. Light apparently suppresses this current by releasing Ca^{++} ions into the rod and cone cytoplasm, where it reduces the Na^+ permeability of their plasma membranes. Several lines of evidence support the central part played by Ca^{++} in visual excitation. Studies of the ATP metabolism of isolated retinal rods also suggest that an essential step in excitation is the rapid phosphorylation of rhodopsin after it absorbs light.

In the area of mammalian physiology, Altland has found that altitude-acclimatized rats (18,000 ft.) showed reduced exercise tolerance when exercised in the cold (5° C). The increased fatigue was due to severe hypothermia (body temperature 27.9 C) and associated decreased metabolism, polycythemia (hematocrit 69), hypoglycemia (80 ± 7 mg/100 ml), reduced serum lactic acid and an increased incidence of myocardial fibrosis. These findings show that available homeostatic mechanisms become exhausted more readily in multiply stressed than in singly stressed animals and that there is a negative cross-adaptation between altitude and other stresses such as exercise and cold.

Because this laboratory relies heavily on sophisticated instrumentation for much of the research, developments in new instruments and techniques play a key role in determining our effectiveness. The acquisition and improvement of several pieces of NMR apparatus have provided a unique investigative facility for NIH and the entire Washington area. Gambhir and Becker, with Ferretti (DCRT), have developed methods for optimizing the use of much of this equipment in pulse-Fourier transform NMR studies, while Gupta, Ferretti and Becker have further developed a useful complement to NMR pulse methods, termed rapid scan Fourier transform NMR.

Jennings, together with personnel from DCRT, has overseen the expansion of the Building 2 time-sharing computer facility to serve one additional instrument, an electron paramagnetic resonance spectrometer. The program library has expanded. The facility is now being used an average of 40 hours per week by one or more users. During busy periods it serves as many as four experiments at once. As use of the computer becomes more intense, plans are being made to transfer some computing tasks to the PDP-10 in Building 12A so as to use the small core memory of the Honeywell H516 more efficiently.

Serial No. Z01-AM-29000-03-LCP
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nuclear magnetic resonance: Developments of new methods and application to molecular structure determination

Previous Serial Number: NIAMDD-LCP-5

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley
Rolf Tschudin
Thomas Pinnavaia
Kensuki Takahashi
Prem N. Gambhir
K. Gurudath Rao

Cooperating Units: T. Clem (DRS-BEIB)
J. S. Cohen (NICHD)
J. A. Ferretti (DCRT)
H. T. Miles (NIAMDD-LMB)
C. N. R. Rao (PL-480 project, Indian Institute of Technology - Kanpur
R. K. Gupta (Institute for Cancer Research)

Man Years

Total:	7
Professional:	5.5
Other:	1.5

Project Description:

Objectives:

1. Elucidation of the forces within and between molecules, especially those of potential biological importance.
2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Major Findings and Future Plans:

Much of our effort during the last year has gone toward the development and improvement of instrumentation and techniques for applying nuclear magnetic resonance (NMR) to the solution of problems in molecular structure and interactions. With the acquisition of several pieces of apparatus this year, we have put into operation three new pulse NMR spectrometers--one, operating at high magnetic field with a superconducting magnet, a second equipped with a very large sample tube capability for ^{13}C NMR, and a third designed for versatility in NMR relaxation time measurements. These acquisitions have had substantial financial support from NICHD, NIDR and NCI, with a strong continuing commitment of resources by DCRT and BEIB-DRS. This Laboratory is rapidly becoming a well equipped, recognized center for NMR research in chemical and biological systems, not only for NIH as a whole, but for the greater Washington area.

In order to use pulse-Fourier transform (FT) NMR methods most efficiently it is important that a number of instrumental and computational parameters which are under the control of the investigator be properly chosen for each experiment. We have computed optimum conditions for many of these factors and are verifying the choices experimentally. (Gambhir, Ferretti, Becker). An alternative (and often useful) procedure to pulse-FT NMR is the rapid scan FT method, developed in part in our Laboratory last year. We have now applied this method to the measurement of relaxation times, where it has some distinct advantages over the widely used pulse-FT methods, and we are examining some of the fundamental limitations of the technique. (Gupta, Ferretti, Becker).

Guanosine-5'-phosphate (GMP) has been known to form gels under certain conditions. We have investigated by proton NMR the interaction between GMP molecules under conditions of concentration and pH where gels do not form but where infrared spectra indicate appreciable association. We have observed, for the first time, that the associated species exchange only slowly with monomer, and with each other, hence give rise to discrete NMR spectra. Studies of chemical shifts, linewidths and relaxation times have provided valuable, but not yet conclusive, information on the structures of the associated forms. ^{13}C NMR studies of GMP are now in progress. (Pinnavaia, Miles, Becker).

Metal ions (e.g., Na^+, Mg^{++}) are known to interact with amides and are believed to affect the barrier to internal rotation in such compounds as N,N-dimethylformamide. We have used NMR methods to investigate quantitatively the changes in barrier height in several amides brought about by complexation with Li^+. (Rao, Becker).

Keyword Descriptors:

Nuclear magnetic resonance, spectroscopy, nucleotides, amides, metal complexes, hydrogen bonding

Publications:

Wasylishen, R. E., Clem, T. R. and Becker, E. D.: NMR chemical shifts of some monosubstituted isothiazoles. Can. J. Chem. 53: 596-603, 1975.

Schwarz, M., Bradley, R. B., McIntyre, H. M. and Becker, E. D.: Proton and ^{13}C NMR spectra of substituted methyl cyanopropionates: Effects of molecular asymmetry. Org. Mag. Res. 6: 625-628, 1975.

Gupta, R. K., Ferretti, J. A. and Becker, E. D.: Spin-lattice relaxation measurements using rapid scan FT NMR. J. Mag. Res. 16: 505-507, 1974.

Tucker, E. E., Clem, T. R., Seeman, J. I. and Becker, E. D.: Carbon-13 NMR relaxation in hydrogen bonded tert-butanol and phenol. J. Phys. Chem. In press.

Wasylishen, R. E., Rice, K. C. and Weiss, U.: NMR evidence for high barrier of conformational inversion in hexahydro-3,3,7,7-tetramethyl-1,2-oxazepine-5-one. Can. J. Chem. 53: 414-418, 1975.

Wasylishen, R. E. and Cohen, J. S.: Spin-lattice relaxation times of imidazole protons and their relevance to nuclear magnetic resonance studies of proteins. Nature 249: 847-850, 1974.

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Gupta, R. K., Pitner, T. P. and Wasylishen, R.: Fourier transform NMR of exchanging chemical systems. J. Mag. Res. 13: 383-385, 1974.

Gupta, R. K., Ferretti, J. A. and Becker, E. D.: Rapid scan Fourier transform NMR spectroscopy. J. Mag. Res. 13: 275-290, 1974.

Serial No. Z01-AM-29001-03-LCP
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Molecular Dynamics

Previous Serial Number: NIAMDD-LCP-6

Principal Investigator: Ira W. Levin

Other Investigators: Robert A. R. Pearce
Robert C. Spiker, Jr. (NIH Research Fellow)

Cooperating Units: S. Abramowitz, National Bureau of Standards
L. Bernstein, University of California, Berkeley, California and Bell Laboratories

Man Years:

Total:	3.5
Professional:	3.0
Other:	0.5

Project Description:

Within the project area of molecular dynamics our objectives are to probe, clarify and interpret the vibrational characteristics of selected moderate to large molecular systems through the following approaches: (a) Analyses of Raman and infrared spectra, (b) determination of reliable intramolecular force fields, and (c) interpretation of molecular charge distribution (infrared and Raman intensity) parameters.

A variety of laser Raman and infrared spectroscopic procedures provide the required experimental data for given vibrational analyses. The intramolecular force field and vibrational intensity computations are necessarily performed on a large digital computer.

We shall divide the results of the past years studies into three subareas: (a) intramolecular force field studies on PF_5, dimethyl ether and 1-methyl uracil, (b) the measurement and interpretation of the absolute infrared vibrational intensities of cyclopropane-d_0 and cyclopropane-d_6, and (c) the Raman spectra of model biological membrane systems.

(a) Intramolecular force field determinations

(1) The stereochemical nonrigidity of PF_5, in which an intramolecular exchange arises between the inequivalent axial and equatorial fluorines through a pseudorotational motion, represents a relatively common aspect of five-coordinate chemistry. An understanding of the potential function and barrier to fluorine exchange in this system can lead to establishing basic stereochemical principles for five-coordinate species. We examined the gas-phase Raman spectrum of the ν_7 fundamental of PF_5 (the vibrational mode leading to pseudorotation) under a spectral resolution approaching 1 cm^{-1}. With the use of a two-dimensional harmonic oscillator basis set and a mixed potential function of the form $V(\rho,\phi) = 1/2\ a\rho^2 - b \cos (3\phi)\rho^3 + c\ \rho^4$, an adequate fit to the ν_7 spectral region was obtained. The barrier to axial-equatorial fluorine atom exchange was estimated to be 1371 cm^{-1} (3.92 k cal/mole). (Levin, Abramowitz, Bernstein).

(2) Since relatively little information exists on either the significance or magnitude of methyl-methyl interaction parameters between vibrations other than the torsional modes, we have extended our earlier studies on these long range potential energy coupling terms to dimethyl ether. Both polycrystalline and mixed crystal Raman spectra of the dimethyl ether $-d_0$ and d_6 species were observed. From a statistically determined 17 parameter modified internal valence force field, a detailed vibrational assignment was proposed for the $-d_0$ and $-d_6$ molecules. This system represents a basic ether linkage for which no complete, consistent vibrational study previously existed. An assignment, in terms of potential energy distributions, was also proposed for the $-d_3$ molecule. For dimethyl ether the significant methyl-methyl coupling term between the anti H-C-S angular displacement coordinates is 0.024 ± 0.011 mdyn -Å, as compared to 0.060 ± 0.013 mdyn -Å for dimethyl sulfide. (Levin, Pearce, Spiker).

(3) In developing the use of modified internal valence force fields for more complex systems, a 30 parameter potential function was determined for the in-plane vibrational modes of 1-methyl uracil. This least-squares adjusted set of converged force constants, based upon the vibrational assignment of E. D. Becker and H. T. Miles, fits 110 observed frequencies for six isotopic species. In these computations the methyl group is approximated by a point mass. Although some modifications in both the force field and assignments are envisioned, these calculations enable one to understand the vibrational properties of this fundamental pyarmidine and allow one to test the accuracy of overlay computations in which a given force field, for example, is required to predict the vibrational properties of a series of pyrimidine derivatives. (Levin, Pearce, Becker).

(b) Since no absolute infrared intensity information exists for strained ring systems, we measured the integrated vibrational intensities of cyclopropane $-d_0$ and cyclopropane $-d_6$ to determine the charge redistribution parameters for this class of molecules. The interpretation of the experimental dipole-moment derivatives $(\partial P/\partial S_j)_0$ proceeded in terms of polar tensors, $(\partial Px/\partial X_\alpha)$ where $x = x,y,z$ for atom α. The polar tensor represents the change in the total dipole moment of the molecule as the set of nuclei undergo small displacements $\underline{r}_\alpha$ ($\underline{r}_\alpha = \underline{i}\Delta x_\alpha + \underline{j}\Delta y_\alpha + \underline{k}\Delta z_\alpha$). Semi-empirical CNDO/2 calculations aided in the analysis. Comparisons are currently being made with the charge distribution parameters of aliphatic systems. (Levin and Pearce).

(c) In applying Raman spectroscopic techniques toward monitoring conformationally dependent vibrations of both natural and model membrane systems, detailed vibrational assignments for dipalmitoyl phosphatidyl choline and structurally related molecules have been proposed for a variety of phospholipid systems. Specifically, vibrational assignments have been clarified for the C-H stretching region (2900 cm^{-1}), C-C and PO_2^- stretching region (1100 cm^{-1}) and the C-N and PO_2 diester stretching region (700-800 cm^{-1}). Emphasis was also placed upon understanding the vibrational characteristics (frequencies and intensities) of membrane vesicles, with diameters of the order of 300Å, as compared to multilamellar aggregates. In particular, phase transitions, curvature and packing effects of vesicles (prepared by sonication) were studied for dipalmitoyl and dimyristoyl phosphatidyl choline bilayer--water systems (Levin and Spiker).

Significance to Biomedical Research and the Program of the Institute:

Since experimentally determined vibrational frequencies and charge distribution parameters reflect the sum of several subtle electronic and nuclear contributions, force field determinations and absolute intensity studies permit both quantitative and qualitative descriptions of the dominant terms and create a link between empirical, spectroscopic information and bond properties.

Raman spectroscopy offers a potentially valuable approach to examining dynamical properties of natural and artificial membrane bilayer assemblies since no membrane perturbing agent is introduced by the technique. This is a particularly crucial in investigating regions of fluidity (order-disorder) in bilayer systems.

Proposed Course of Project:

1. Development of the techniques of applying modified internal valence force fields toward interpreting the potential and kinetic energy coupling effects in increasingly more complex molecular systems. Investigations will continue on the large amplitude, pseudorotational effects of the pentacoordinated AsF_5 and VF_5 systems. With the advent of versatile interactive normal coordinate computer programs, studies will be initiated to monitor conformational changes in both the hydrocarbon and head group regions of lipid bilayers in terms of specific structure models.

2. Investigations of the vibrational behavior of both vesicle and multilamellar bilayer systems will continue with emphasis on (a) compositional bilayer asymmetries introduced by the effects of surface curvature and (b) lipid-protein interactions by means of resonance Raman techniques using tunable dye laser procedures.

Keyword Descriptors:

Modified internal valence force fields, absolute infrared intensities, Raman spectra of model lipid bilayer assemblies, pseudorotation in pentacoordinated species.

Publications:

Levin, I. W.: Laser Raman Spectroscopy. In Turk, A., Johnston, Jr., J. W., and Moulton, D. G. (Ed.): *Human Responses to Environmental Odors*. New York, Academic Press, Inc., 1974, pp. 45-120.

Levin, I. W., Pearce, R. A. R., and Spiker, R. C. Jr.: Intramolecular potential function and methyl-methyl coupling of crystalline dimethyl sulfide. *Spectrochimica Acta* 31A: 41-55, 1975.

Wiberg, K. B., Sturmer, D., Lewis, T. P., and Levin, I. W.: Vibrational spectrum of bicyclo (1.1.1) pentane. *Spectrochimica Acta* 31A: 57-73, 1975.

Levin, I. W., and Pearce, R. A. R.: Intramolecular force field calculations methods and applications. In Durig, J. R.: *Vibrational Spectra and Structure*, Vol. 4. Elsevier Scientific Publishing Co. In press.

Bernstein, L. S., Kim, J. J., Pitzer, K. S., Abramowitz, S., and Levin, I. W.: Potential function for the ν_7 vibration of phosphorus pentafluoride. *J. Chem. Phys.* In press.

Spiker, R. C. Jr., and Levin, I. W.: Raman spectra and vibrational assignments for dipalmitoyl phosphatidylcholine and structurally related molecules. *Biochim. Biophys. Acta.* In press.

Serial No. Z01-AM-29002-02-LCP
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study.

Previous Serial Number: NIAMDD-LCP-8

Principal Investigator: Ulrich Weiss

Other Investigator: Kunitoshi Yoshihira

Cooperating Units: Dr. James M. Cook, Department of Chemistry, University of Wisconsin, Milwaukee

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution of a variety of naturally occurring substances of chemical, biosynthetic, or biological interest. Study of instrumental techniques used in such work. Corroboration of chemical structure by total synthesis. Synthesis of compounds of medical or biological interest.

Methods Employed:

Standard procedures of organic chemistry, and instrumental methods (UV, IR, NMR spectroscopy). For measurement of optical rotatory dispersion and circular dichroism, the Cary 60 spectropolarimeter and its CD attachment are used.

Major Findings and Future Plans:

The chemical structure of the antibiotic chrysomycin is being studied by Dr. Yoshihira. This antibiotic was isolated as lemon-yellow crystals in 1955 by the late Dr. Frieda Strelitz at the Research Laboratory of the New York Botanical Garden, during a survey of molds for action against bacteriophage. It has never been studied further, and the mold from which it had been isolated has mutated and does not produce chrysomycin any longer. Through the courtesy of colleagues at the Botanical Garden, I have obtained the entire supply left by Dr. Strelitz; the amount ought to be sufficient for structure elucidation by modern instrumental methods. Besides its being active against many bacteriophages, chrysomycin has marked activity against certain bacteria and several molds and it shows low toxicity in mice.

In our work, the available material was shown to be inhomogeneous, consisting mostly of chrysomycin A together with minor amounts of a new, related substance, chrysomycin B. The study of the chemical structure of these compounds is being pursued.

The analgesic action of the 1-fluorocodeine prepared last year by Dr. Lousberg (see report for 1974) has been found by Dr. E. L. May (LC-NIAMDD) to be very close to that of codeine itself. Furthermore, Dr. W. Klee (LGCB-NIMH) has established that the two compounds are bound to about the same extent by the opiate receptor from the central nervous system. It appears, therefore, that substitution of the hydrogen in position 1 of codeine by fluorine has little effect on pharmacological action or receptor binding. Consequently, the long-known diminished analgesic action of 1-chloro- and 1-bromocodeine must be due to the bulk of the halogen atoms rather than to any electronic effect upon the aromatic ring; for fluorine, the latter effect should be maximal, while its space requirement, in contrast to those of Cl and Br, is hardly higher than that of hydrogen. Our results thus provide information on the way in which morphine-type analgesics interact with the receptor site.

Unexpected experimental difficulties in the preparation of 1-fluorocodeine have made it impossible to carry out the planned synthesis of the isomeric 2-fluorocodeine within the short time available to Dr. Lousberg during his visit to NIH in 1974. It is hoped that it will be possible to resume his work on this problem, and to prepare the complete series of all the 1- and 2-halocodeines and -morphines.

The study of the two new products of the reaction of dimethyl β-ketoglutarate and glyoxal in aqueous solution at room-temperature has been completed. Some of the further transformations of one of them, dimethyl 1,10-dihydroxy-tetracyclo$[6.5.0.0^{2,10}.0^{3,7}]$tridecane-5,12-dione 2,8-dicarboxylate, have yielded unexpected and interesting new substances.

The reactions of dimethyl β-glutarate with glyoxal and other 1,2-dicarbonyl compounds under "physiological" conditions are being explored further in cooperation with Dr. James M. Cook and his group at the University of Wisconsin, Milwaukee. The reactions occurring in these systems are of interest as model reactions for biosynthetic processes, and their synthetic potentialities are almost unlimited. There is reason to hope that this work, which is to be continued, will lead to compounds of therapeutic interest.

The expectation of pharmacological study of hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5 by Prof. Ulf von Euler, Stockholm, has not materialized, perhaps because of his advanced age. It is hoped that it will be possible to have this study carried out elsewhere; the activity of this compound, observed already in 1897 but never explored further, may be related to that of curare, and hence well worth further examination; curarimimetic action of a compound of this structure would be unexpected, and the marked preference for one, evidently strongly non-planar, conformation could give information on connections between molecular shape and pharmacological activity.

Keyword Descriptors:

Chrysomycin antibiotics, structures; 1-fluorocodeine, analgesic action, receptor-binding; biomimetic syntheses

Publications:

Lousberg, R. J. J. C., and Weiss, U.: The analgesic action of 1-fluorocodeine. Experientia 30: 1440, 1974.

Wasylishen, R. E., Rice, K. C., and Weiss, U.: Nuclear magnetic resonance evidence for high barrier of conformational inversion in hexahydro-3,3,7,7-tetramethyl-1,2-oxazepine-5-one. Can. J. Chem. 53: 414-418, 1975.

REPORTED IN PRESS - 1974

Edwards, J. M., and Weiss, U.: Root pigments of Lachnanthes tinctoria. Phytochemistry 13: 1597-1602, 1974.

Weiss, U., Tillack, J. V., and Kennard, C. H. L.: Stereochemistry of dihydrometacodeinone. X-ray crystallographic proof of the configuration and conformation derived from the nmr spectrum. Proc. Roy. Aust. Chem. Inst.: 106-108, 1974.

Serial No. Z01-AM-29003-08-LCP
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest

Previous Serial Number: NIAMDD-LCP-7

Principal Investigator: Gerald M. Shean

Other Investigators: None

Cooperating Units: Karl Sollner (Scientist Emeritus)

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives:

To prepare and study well-defined synthetic porous and liquid ion-exchanger membranes and membrane systems that duplicate various in vivo membrane phenomena such as cell potentials, electrolyte distributions and water movements. To provide a rational physico-chemical basis for understanding the mechanisms that underlie these phenomena.

Methods Employed:

Conventional electrochemical, spectral, and flame analytical methods as well as potentiometric measurements were employed.

Major Findings:

The concept of accumulating both cations and anions simultaneously based on Donnan equilibria has previously been developed in the laboratory. (Sollner). An experimental verification has now been made of this concept using a liquid ion-exchanger charge-mosaic membrane consisting of anion permeable and cation permeable portions. The accumulation of a salt inside the experimental cell was coupled with the movement of another salt to the outside of the cell. As an example, a cell consisting of the following was made:

Outside solution: maintained at 10^{-3} N NaAc, 10^{-4} N KCl. Mosaic membrane: .1 M triocty methyl ammonium chloride in o-xylene membrane in parallel with .1 M potassium dinonylanaphthalene sulfonate in o-xylene membrane. Inside solution: maintained at 10^{-2} N KCl. At the end of the experiment the inside solution had become .032 N in regard to acetate and .028 N in regard to sodium, which is an accumulation of about 30 times over their concentrations in the outside solution. Ideally the NaAc should have reached 10^{-1} N inside the cell according to the Donnan equilibria. The difference was due to leakage of ions to the outside solution.

Charge-mosaic membranes made with porous and liquid ion-exchanger membrane portions showed that it is not necessarily valid to assume membrane selectively from membrane potential data or vice versa. By placing in parallel a porous cation permeable sulfonated polystyrene membrane portion of 1,200 ohms with a stirred liquid anion permeable secondary amine in o-xylene membrane portion of 49,000 ohms a mosaic membrane potential resulted that indicated that the mosaic membrane was cation selective. (Cations of the more potentiogenic side of the membrane impressed their charge on the opposite side.) However analyses of the solutions showed that ten times as many anions had crossed the membrane as cations. This was due to the fact that the electrical resistance porous ion-exchanger membranes is directly related to the ionic permeability while with liquid ion-exchanger membranes the electrical resistance is not a direct measure of the ionic permeability because the latter primarily occurs in the uncharged state via carriers rather than ionically. Stirring the liquid membrane portion greatly increases the anion permeability rate without affecting the membrane electrical resistance.

Significance to Biomedical Research and the Program of the Institute:

Important to all living systems are the mechanisms that regulate the permeabilities of electrolytes, non-electrolytes and water through membranes. Understanding of the complex membrane phenomena in living cells and tissues is helped by information gained from simple membrane model systems that can reproduce some of the phenomena that occur in living systems. Model studies indicate the possibility of easy explanations for phenomena that have previously been thought of as very complicated. The accumulation of electrolytes against concentration gradients in models that rely only on known physico-chemical principles reported here is one example.

The occurence of electrical potentials across living cell membranes is wide-spread. How they arise and change in magnitude are subjects of extensive research which can be aided by electromotive experimentation employing the well characterized, synthetic membranes used in this project. Steady state and equilibrium potentials as well as those from fast degrading systems can be studied with a facility that is lacking with most living cell membranes. The possible transference of the information gained with the synthetic membranes to the living membranes is obvious.

Proposed Course of Project:

Continuation of model building with permselective porous and liquid membranes. The assembling and studying of mosaic membrane systems that show electrolyte accumulations close to those predicted by Donnan equilibria. Studying interactions of electrolyte, non-electrolyte and water flow, across membranes with the object of understanding a variety of permeability, electromotive and osmotic effects.

Keyword Descriptors:

Membranes, ion-exchange, potentials, transport, ions, accumulation, bi-ionic, models, selectivity, permselective.

Serial No. Z01-AM-29004-06-LCP
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Photochemical reactions and their mechanisms

Previous Serial Number: NIAMDD-LCP-9

Principal Investigator: Herman Ziffer

Other Investigator: Kuninobu Kabuto, NIAMDD-LCP

Cooperating Unit: Paul S. Engel, Rice University

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

In order to permit a more rational use of photochemical reactions in the synthesis of compounds that are different to prepare by conventional methods, a better understanding of the mechanism and stereochemical requirements of these reactions is necessary. Our studies on photochemical isomerizations have been primarily concerned with those of β,γ-unsaturated ketones. We have completed one aspect of our investigations of the β,γ-unsaturated ketones of cyclopropyl ketones (oxa-di-π-methane rearrangement) by demonstrating that the stereochemistry about the α-carbon in these compounds is retained during the isomerization. In another study in collaboration with Dr. Engel (Rice University, Houston, Texas) we have established that the triplet energy of the reactive excited state is significantly higher (74-78 kcal.) than the 61-74 kcal. previously reported. This difference in triplet energies makes it understandable why it is necessary to use acetone (80 kcal.) rather than benzophenone (68 kcal.), or acetophenone (74 kcal.) as a sensitizer for this isomerization. In addition, a study on the excited state properties of 3,4,5,6,7,8-hexahydronaphthalen-2(1H)-one yielded data enabling us to estimate the lifetime (4 nsec.) of the triplet. It is planned to extend our studies on the oxa-di-π-methane rearrangement by determining if the use of a high energy optically active sensitizer can be used to form optically active photoproducts. Although there are very few examples of photochemical asymmetric synthesis, the short lifetimes for the excited species and the stereospecificity observed for this reaction suggest that the products could be optically active.

In the course of an earlier study of β,γ-unsaturated ketones, photoepimerization of a β,γ-unsaturated ketone was noted. The reaction was rationalized as proceeding via a diradical intermediate which ring closed in a stereospecific manner, i.e., a cis 6/5 ring junction isomerized to a trans 6/5 junction. The reaction is extremely valuable since it alters the stereochemistry from a more stable to a less stable (ground state) one. In order to test aspects of the above mechanism, i.e., ring opening to a relatively long lived di-radical which ring closes in a stereospecific manner, we wish to study some β,γ-unsaturated ketones that contain 6/6 ring junctions. The above mechanism requires that the stereochemistry at a 6/6 ring junction change from cis to trans, i.e., opposite to that observed in the 6/5 example.

In addition to the above mentioned studies of β,γ-unsaturated ketones, we are interested in uncovering new photochemical reactions and will examine the photochemistry of other compounds.

Keyword Descriptors:

Photochemistry, β,γ-unsaturated ketones

Publications:

Engel, P. S., Schexnayder, M. A., Phillips, W. V., Ziffer, H., and Seeman, J. I.: Excited state properties of 3,4,5,6,7,8-hexahydronapthalen-2(1H)-one. Tetrahedron Letters 1157-1160, 1975.

Serial No. Z01-AM-29005-01-LCP
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Titles: Asymmetric synthesis; Structure, stereochemistry and NMR

Previous Serial Number: NIAMDD-LCP-9

Principal Investigator: Herman Ziffer

Other Investigator: Kuninobu Kabuto, NIAMDD-LCP

Cooperating Units: Donald M. Jerina, NIAMDD-LC
Herman J. C. Yeh, NIAMDD-LC
D. T. Gibson, University of Texas
Robert J. Highet, NHLI-IR-CH

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Asymmetric Synthesis

In the course of some studies establishing the absolute stereochemistry of aromatic metabolites (dihydro diols) the severe limitations associated with working with the small quantities of material available from enzymatic reactions became apparent. In order to overcome this problem we started a program on asymmetric synthesis. We have successfully demonstrated that substantial (several hundred mgs) amounts of optically active material (tetrahydro diols) suitable for determining the absolute stereochemistry of these compounds and other studies can be prepared. In the initial approach, cis and trans 3-phenyl 1,2-cyclohexane diols were obtained by reduction of α-acetoxy ketones with a chiral reducing agent (lithium aluminum hydride: darvon (1:2) complex). The resulting cis and trans diols were formed with enantiomer excesses of approximately 25-40%. The absolute stereochemistry of these compounds is currently under investigation.

Suitable procedures for the preparation of α-acetoxy ketones in the phenanthrene series have been developed and the absolute stereochemistry of the resulting diols are under investigation.

Structure, Stereochemistry and NMR

In order to confirm the relative stereochemistry assigned to a number of diols it was necessary to prepare all possible isomers. The availability of the isomeric 3-methyl 1,2-cyclohexane diols, the cis, cis isomer was previously unknown, prompted us to look for correlations between the chemical shifts of the various carbons and their stereochemistry in the ^{13}C nmr spectra of these compounds. The results of this investigation were reported this year. We have also recorded and analyzed the ^{13}C nmr spectra of the isomer 3-phenyl 1,2-cyclohexane diols prepared for analogous reasons and plan to record and analyse the ^{13}C NMR spectra of the tetrahydrophenthrene diols.

Since the optical properties of most of the compounds prepared by asymmetric synthesis are unknown, it was necessary to develop analytical methods for establishing the enantiomeric excess produced in the asymmetric step. We have been successful in doing so by examining the proton nmr spectra of the above mentioned diols in the presence of optically active shift reagents. Once the absolute stereochemistry of more diols is established we plan to prepare derivatives of the diols with an optically pure reagent and look for empirical correlation between the relative chemical shift of proton, carbon and fluorine resonances in the various diastereomers and the absolute stereochemistry at the chiral centers.

Keyword Descriptors:

Metabolites, diol syntheses, NMR ^{13}C and proton

Publications:

Ziffer, H., Seeman, J. I., Highet, R. J. and Sokoloski, E. A.: Carbon-13 nuclear magnetic resonance characteristics of 3-methylcyclohexane-1,2-diols. J. Org. Chem. 39: 3698-3701, 1974.

Serial No. Z01-AM-29006-05-LCP
1. Chemical Physics
2. Spectroscopy and Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Relationship Between the Nuclear and Electronic Configurations of Molecules and Their Spectroscopic and Dynamic Properties.

Previous Serial Number: NIAMDD-LCP-11

Principal Investigator: Elliot Charney

Other Investigators: Joan Rosenfield

Cooperating Units: Kiwamu Yamaoka, University of Hiroshima, Japan

Man Years:

Total:	3.5
Professional:	2
Other:	1.5

Project Description:

This report covers projects in two different but allied fields: (1) Molecular chiral phenomena and (2) macromolecular structure and dynamics. The object of the first project is to try to increase our understanding of the nature of molecular chiral phenomena in general and in particular to see the extent to which quantum mechanical interpretation of molecular states provide a detailed explanation of the chirality of small molecules or individual chromophores. The objectives of the investigations into macromolecular structure and dynamics are to determine the molecular structure of polymers, biological polymers in particular, in determining their physical properties—and the converse as well. The special experimental methods employed have been described in previous reports and the theoretical methods are standard in the field.

1. Molecular Chiral Phenomena

a) There is no intermediate monograph or text available to provide a comprensive and comprehensible explanation of the underlying basic electromagnetic and quantum theories of molecular chiral phenonmena, that is, a text which will bring the knowledge of the theory available in this field to biochemists and organic chemists. In fact, there is a noticeable gap in the scientific literature; there are only about six extent texts (five in English) ranging from the first published in 1935 to the most recent published in 1973. The latter is an exposition of the theory in terms suitable for theoreticians and the others are primarily compendia of experimental information. As part of this project a monograph to fill this gap is in preparation.

b) The α-diketones are a large class of molecules whose lowest molecular weight representative is glyoxal, CHOCHO. This molecule is not chirally active but many of its higher molecular weight representatives are. The electronic description of the excited states of this molecule are still in question. In 1971, we [Dr. Lin Tsai and the principal investigator] published the results of an exhaustive spectroscopic and simple theoretical investigation of the chirally active α-diketone, camphorquinone, which shed new light on the lower excited states of this system. The publication, since then, of the results of some seven or eight new investigations of this system, have led us to attempt a more sophisticated theoretical treatment (CNDO/CI) of this treatment which will include the calculation of the magnetic as well as the natural circular dichroism. The first calculation of the natural CD has produced a result which is somewhat different from our original interpretation but is in accord with the experimental results on states at higher energies that we were unable to interpret originally. This calculation was done for the camphorquinone geometry. We are preparing to repeat the calculation for another α-diketone of much different geometry, camphenilone.

c) In addition to the collaborative work on the α-diketones, Dr. Joan Rosenfield is continuing the research she started at the University of Minnesota on the vibronic states of benzene and their contributions to the magnetic circular dichroism as well as the natural circular dichroism. The three lowest energy electronic UV absorption bands of benzene have long been analyzed as arising from transitions to two forbidden (B_{2u} and B_{1u}) and one allowed (E_{1u}) excited states. The newest calculations of magneto-optical parameters using the CNDO/S-CI method suggest that a feature in the magnetic circular dichroism vapor spectrum of benzene on the low energy edge of the E_{1u} absorption may arise from still a fourth state, an electric-dipole allowed, out of plane polarized A_{2u} state. With this in mind, other calculations are underway on the B_{1u} transition of benzene about which there is still considerable uncertainty.

2. Macromolecular Structure and Dynamics

The orientation of large polymeric molecules in pulsed electric fields and their disorientation when the field is turned off can be studied using monochromatic polarized light. These experiments are designed to obtain several types of information, including the molecular conformation or structure in solution (from the dichroism, which is the difference between the changes in extinction of the light polarized parallel and perpendicular to the electronic field when the molecules are completely orientated in the field) and the dynamics of intramolecular reorganization and of rotational disorientation (from the decay of the dichroism at the end of the field pulse). Of the latter two, the intramolecular dynamics is at present available to our measurements only for motions with relaxation times of about 5 x 10^6 seconds or slower. Most of the intramolecular dynamics occurs faster than that and is therefore a subject for future studies as the time resolution of the apparatus is improved, or by other methods. Experiments are currently underway to extract structural information. We have found that in glycerol solutions of calf-thymus DNA (in the absence of Mg^{++} ion) no effect of the glycerol on the conformation of the nucleic acid is observed up to glycerol concentrations of 25 volume percent. This is part of a study aimed at understanding the biochemical effects of glycerol in DNA transcription in λ phage. Future studies depend on the ability to obtain short DNA segments containing the gal operon of λ phage where the biochemical effects have been manifest. In addition to this, a project on the detailed solution dichroism properties of small segments of DNA, co-sponsored by the Japan-USA Cooperative Science Program, is underway.

Proposed Course of the Project:

In addition to the completion of the projects described above as underway, it is proposed to continue with several new or related investigations of molecular dynamics by electric dichroism and/or birefringence. In particular, we expect to examine in detail the dichroism and relaxation of polypeptides in which a freely rotating link is incorporated approximately in the center of the longer polymer. It is our expectation that in the presence of the electric field these molecules will orient as though they were a continuous rod. It remains to be seen whether these expectations will be correct and to examine in detail the relaxation phenomena which have a direct bearing on earlier investigation of the relaxation of polyriboseadenylic acid.

Keyword Descriptors:

Molecular chiral activity, electric field orientation, molecular relaxation

Publications:

Glaubiger, D., Kohn, K. W. and Charney, E.: The relaxation of anthamycin with DNA. IV. Properties of the complex. Biochem. Biophys. Acta 361: 303-311, 1974.

Charney, E. and Milstein, J. B.: Electric dichroism of polyriboseadenylic acid. Biopolymers. In press.

Charney, E.: Electro-optics in the infrared region. In O'Konski, C. T. (ed.): Molecular Electro-Optics. New York, New York, Marcel Dekker. In press.

Serial No. Z01-AM-29007-04-LCP
1. Chemical Physics
2. Spectroscopy and Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure and interaction of paramagnetic biomolecules

Principal Investigator: Hideo Kon

Other Investigators: Mitsuo Sato

Guest Workers: Yoshikazu Ioki (Aug. 16 - Oct. 15)
Amy Rispin (Sept. 15 - May 31)

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

The fundamental objective of this project is to clarify the structure-function relationship of the paramagnetic centers existing in various biological systems. Of the two major categories of the paramagnetic molecules, i.e. organic or inorganic free radicals and the transition metal ions, more emphasis has been placed in this project on the biomolecules associated with the paramagnetic centers of the latter type, mainly because the metal ions are usually incorporated as part of the integral biological machinery of metabolism rather than the product or the intermediary species to be found in the metabolic process.

Two apparently different approaches are taken depending upon the level of the present knowledge of the system to be studied. When the intrinsic importance of the metal ions in the biochemical process is established and its structural environment being defined fairly well, the project aims at obtaining the details of the energy level structure and of the mode of interaction with ligand (substrate), by making full use of proper model systems in which more precise measurements under wider variety of conditions are feasible than with the actual biomolecules. The information thus obtained may be fed back to help interpreting the data on the biomolecules which in most cases are less clear and less amenable to interpretation by themselves. In the systems in which the intrinsic importance of the metal ion is suspected but not established the biomolecules are subjected to the observation to determine if the correlation exists between the spectroscopic signal and the known biochemical reactions.

Electron paramagnetic resonance absorption (epr) has unique advantage for studying the paramagnetic molecules, and is used as the principal experimental technique in conjunction with other spectroscopic methods. For most of the metal ions the observation in the sub-nitrogen temperature range is essential, and the cryogenic techniques are heavily used. The ligand field calculations including the use of a computer is an important theoretical tool for analyzing the spectroscopic data to obtain quantitative information on the electronic structure of the metal ion in the particular environment.

Major Findings:

1. (With Dr. Sato)

The epr spectrum of the high spin heme Fe(III) in cytochrome P450 shows a characteristic absorptions with g-values near 8, 3.7, and 1.7, indicating an unusually high rhombic magnetic environment for Fe ion. Attempts have been made in this project to find a model system to simulate the epr spectrum of P450. We have found that epr absorptions corresponding to the structures with various degrees of large rhombicity can be observed when tetraphenylporphine Fe(III)X, where X is an anion, is coprecipitated with a large excess of free tetraphenylporphine (TPP). The crystalline precipitate is apparently a mixture of several species each with different degree of rhombicity. The highest rhombicity observed in this system is almost the same as in P450. In this particular species the F^{19} hyperfine splitting is absent in contrast to other species, the fact which, together with other evidence, strongly indicates that in this highly distorted state the fifth ligand may be severely distorted or displaced, making the Fe(III) virtually tetracoordinated. The origin of the rhombicity may be explained as basically due to the crystal packing force. These results demonstrate that in spite of the widely adopted hypothesis the -SH ligand for the fifth coordination site is not a necessary condition for the unusual rhombicity in P450.

2. (With Dr. Sato)

It has been predicted on theoretical basis that in the highly rhombic Fe(III) porphines, such as P450, the epr transitions within the middle (excited) Kramers doublet may be observed, but no such observation has yet been made. We have succeeded to detect, in the model systems mentioned in 1, several sets of epr lines the g-values of which are in agreement, in the first order, with the predictions. The slight discrepancy may be ascribed to the higher order Zeeman perturbation. The possibility of estimating the zero-field split (between the Kramers doublets) by making use of this deviation is under investigation. This observation was made possible because of the model system with much better resolution in the epr spectra, and makes it a more meaningful effort to look for similar transitions in P450 itself.

3. (With Dr. Sato)

Co(II) ion has been used lately in some heme proteins to replace Fe for probing the heme environment. Thus the prosthetic group Co(II) porphyrin has been known to be in the low-spin ^2A, state with well-defined epr. We have found, however, that there are two forms of Co(II)tetraphenylporphine in polycrystalline state; the form A which shows the characteristic epr of ordinary low-spin ^2A state, and the form B which does not show any epr down to 1.3°K. Two forms are interconvertible by heat treatment (A→B) and recrystallization (B→A). No solvent molecules are involved. A collaboration for the magnetic susceptibility (X) measurement of these two forms, (first with Dr. A. Thorpe, Physics Department, Howard University, and recently with Dr. A. Tasaki in Osaka University, Japan) revealed that the form B shows a transition in X^{-1}/T curve at ∿15°K, below which the system follows Curie law with effective magnetic moment of 3.5 Bohr magnetons (theoretical value for $S = 3/2$ is 3.87). This result strongly indicates that the ground state of the form B is described by some high-spin state. Ligand field analysis including seven L-S states is in progress.

4. Dr. D. W. Nebert (CH-LBS)

It has been a common practice in the biochemical study of mono-oxygenases, in which cytochrome P450 is one of the active components, to use the optical absorption difference spectrum for studying the mode of substrate P450 interaction. The substrates are classified on that basis as types I and II, which, respectively, are assumed to shift the spin state of Fe(III) in P-450 toward the high- and the low-spin state. A confusing picture emerged sometimes, however, when the conclusion was drawn solely on the basis of the difference spectrum. We have made a detailed study of the relationship between the trend in the difference spectrum and the actual high and low-spin content obtained by epr. The results demonstrated that the epr data in general support the classification of the substrates, but when the preconditioning of the animal was significant or when the type I and II substrates are simultaneously used, only the epr data can provide a correct basis for the interpretation of the difference spectrum. The epr data also showed that some substrates cause the shift between the two different low-spin states, which the optical method can hardly distinguish.

Other Collaborative Efforts:

Since we have the only capability for conducting the epr study of the metal containing systems under the cryogenic conditions, we often make collaborative effort with the investigators in other institutes within a certain limit and when some meaningful results can be expected.

1. Dr. Herman J.C. Yeh (A-LC)

Hemin(Fe(III)) cyanide complex was studied at 10°K. The evidence was obtained which corroborates Dr. Yeh's conclusion by nmr study that there are two low-spin species in this complex.

2. Dr. Gordon Guroff (CH-LBS)

A preparation of phenylalanine hydroxylase was subjected to epr examination at 7°K. Some of the absorptions which can be assigned to Fe(III) ion, responded favorably with biochemical reagents. A further purification of the enzyme is under way in Dr. Guroff's laboratory.

3. Dr. Edward Kang (American National Red Cross) and Dr. C. B. Storm (Howard University)

Carboxypeptidase with the Zn^{2+} ion replaced by $Co^{(II)}$ was studied at 9°K. The epr signal at $g \sim 4$ was attributed to Co(II) ion.

4. Dr. E. Weinbach (I-LPB)

A protozoa ENT-AMOEBA hystoritica, which causes Amebiasis in human beings, has no cytochromes in its electron transport system. Biochemical analysis indicated a high iron-protein content, which may indicate that the electron transport is carried out, quite exceptionally, by iron-sulfur protein only. Epr study at 9°K indeed showed an absorption at g=2.03 with biochemical behavior characteristic to the iron-sulfur protein. Further study will be made after purification of the protein.

5. Dr. J. A. Berzovsky (A-LCB)

Oxyhemoglobin emulsion in Freund's adjuvant of rabbit which is used for the Hb-antibody formation was subjected to epr observation to find out if and to what extent the hemoglobin is oxidized to methemoglobin in either high-spin or low-spin form during the emulsification process. The result showed that such oxidation is very minor ($\lesssim 5\%$) and the product is almost exclusively in the high-spin form.

Short term future plan.

In addition to continuing the current investigation, some additional effort will be made to study if the free radical formation is involved in the metabolic process mediated by microsomal P450 and to find the relationship between the spin state and the free radical yield.

We are planning to acquire the capability of the magnetic susceptibility measurement which mutually supplements with the epr information on the metal ion in elucidating the fine structure in the electronic states.

Keyword Descriptors:

Transition metal ions, epr, ligand field theory.

Publications:

Sato, M. and Kon, H.: The high spin Fe(III) tetraphenylporphine with unusually large rhombic character. Inorg. Chem. In press.

REPORTED IN PRESS - 1974

Kon, H.: An interpretation of the three line epr spectrum of nitric oxide hemoproteins and related model systems: The effect of the heme environment. Biochim. Biophys. Acta 379, 103-113 (1975).

Serial No. Z01-AM-29008-04-LCP
1. Chemical Physics
2. Spectroscopy and Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Electronic and molecular structural investigations

Previous Serial Number: NIAMDD-LPC-8

Principal Investigator: Ruth McDiarmid

Other Investigators: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

To describe the electronic and molecular structures of the ground and excited states of simple molecules (1) for the information they provide about these molecules themselves, (2) to assess the validity of other means of determining these structures (e.g. theoretical calculations) and (3) as models to aid in interpreting the less detailed data obtainable for more complex molecules. Within these objectives, the specific subject of investigation changes in time, old projects being finished and new projects being initiated.

Methods:

The main technique employed is the measurement and analysis of electronic absorption spectra, predominantly in the ultraviolet. As needed in the course of research, other techniques are employed, including: (1) the production of unstable molecules by pyrolytic techniques, (2) the calculation of energy levels and geometries and (3) the generation of calculated spectra to aid in the analysis of the experimental data.

Current Project:

The current project is the analysis of the spectrum of trans-1,3-butadiene. Butadiene is the simplest polyene, hence the one most amenable to theoretical treatment. Subsequent to the demonstration that a previously unsuspected forbidden transition lay lower in energy than the first $\pi \rightarrow \pi^*$ transition in one specific polyene, several theoretical investigations were initiated to see how general is the phenomenon of low-lying forbidden transitions in polyenes. Our investigation of butadiene was initiated for the same purpose.

A previously unobserved electronic transition was found at slightly higher energy than the main $\pi \rightarrow \pi^*$ system at 2150Å, which was shown, with the aid of the simultaneous analyses of the spectra of 1,1,4,4-butadiene-d_4 and butadiene-d_6, to be forbidden. The true origin of the electronic transition was located and its vibrational structure analyzed.

Measurement and analysis of the remaining spectrum to 1350Å revealed that (1) several of the previously reported transitions were misassigned, (2) a region of diffuse absorption around 1450Å was not previously observed and (3) the previously reported absorption continuum around 1700Å might arise from the accidental "piling up" of three unrelated, discrete electronic transitions. The electronic spectrum was shown to be composed of Rydberg transitions and excited state vibrational transitions built on the Rydberg origins.

An attempt is currently underway to determine the direction of the electronic transition moment in one or two of the electronic transitions. When this has been achieved, we anticipate that this investigation of the spectrum of butadiene will be complete.

Significance to Biomedical Research and the Program of the Institute:

This research project seeks to understand the electronic changes a molecule undergoes on absorbing light and, more significantly, the structural alterations that a given electronic change can cause. This study can, if fruitful, provide a basis for a physical interpretation of the relative spectroscopic results obtained in other, more general studies.

Of butadiene investigation: We have now shown that the existence of a forbidden, hence very weak, energy level near the main $\pi \rightarrow \pi^*$ absorption is not unique to one specific polyene. By extrapolation, an analogous transition is expected in other polyenes. Thus, this investigation of the spectra of a model compound bears directly on the understanding of the energy levels of important visual pigment retinene, a specific, more complicated polyene.

Proposed Course of Project:

1. This year we intend to investigate the ultraviolet absorption spectra of several unstable molecules of interest. Initially we hope to generate, observe and analyze $H_2C{=}S$ and HN=NH.

2. There remain several unresolved aspects of the ongoing ethylene investigation, which we hope to resolve.

3. A more formal investigation of the effect of electronic excitation on torsional barriers will be initiated.

Keyword Descriptors:

Ultraviolet spectroscopy, energy levels, Rydberg series, butadiene.

Publications:

McDiarmid, R.: Assignments in the ultraviolet spectra of MoF_6 and WF_6. J. Chem. Phys. 61: 3333-3339, 1974.

McDiarmid, R.: On the forbidden 2000Å transition of *trans*-1,3-butadiene. Chem. Phys. Lett. In press.

REPORTED IN PRESS - 1974

McDiarmid, R.: On the 1700Å transition of dimethyl ether. J. Chem. Phys. 60: 4091-4092, 1974.

McDiarmid, R.: C-O torsional vibrations in the 1900Å transition of diethyl ether. J. Chem. Phys. 60: 3340-3341, 1974.

McDiarmid, R.: Assignments of Rydberg and valence transitions in the electronic absorption spectrum of dimethyl sulfide. J. Chem. Phys. 61: 274-281, 1974.

Serial No. Z01-AM-29009-02-LCP
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Sickle Cell Disease

Previous Serial Number: NIAMDD-LCP-12

Principal Investigators: William A. Eaton, James Hofrichter and
Philip D. Ross (NIAMDD-LMB)

Man Years:

Total: 2.5
Professional: 2.5

Project Description:

Objectives:

The major aim of this work is to develop agents for the treatment of sickle cell disease through physico-chemical studies on the gelation of hemoglobin S.

Methods Employed:

We have developed a variety of techniques to study the gelation reaction, including scanning microcalorimetry, optical birefringence, light scattering, water proton nmr linewidths, and sedimentation methods.

Major Findings:

It is known from the work of others that gelation consists of a highly concerted polymerization of hemoglobin S molecules into fibers, and the alignment of these fibers to form some kind of crystalline phase. The aligned fibers rigidify the red cell and produce the distortion referred to as sickling. Our work represents the first kinetic study of the gelation reaction. We have found that the time course of gelation is very unusual. When gelation of hemoglobin S is thermally induced, the reaction does not start as soon as the temperature is raised. Prior to the onset of the reaction there is a delay period, during which there is no measurable fiber formation. A delay period has been observed with all of the techniques mentioned above. Once fiber formation does start, it proceeds rapidly, and is over 90% complete in a time approximately equal to the delay time. By varying the temperature or total deoxyhemoglobin S concentration, the delay time can be made to vary from less than a second to many hours, or even days. For example, changing the final temperature from 30°C to 15°C increases the delay time by a factor of 1000. The sensitivity to concentration is even more striking. The delay time is inversely proportional to about the 30th power of the total deoxyhemoglobin S concentration. This means that a 20% decrease in the concentration will decrease the delay time by a factor of 800. To our knowledge this is the largest concentration dependence ever measured for a molecular process.

Dr. Hofrichter has developed a theoretical model which accounts for the major features of the gelation kinetics. The rate-limiting process in his model is nucleation of individual fibers. Aggregation begins via a series of thermodynamically unfavorable bimolecular steps. The aggregate grows by stepwise addition of monomers until a critical nucleus is formed; polymerization then proceeds by a series of thermodynamically favorable monomer addition steps. The solution of the steady state rate equations shows that the rate of polymer formation can depend on a very high power of the monomer concentration, and that the apparent activation energy is composed of a sum of energy terms, the largest contribution arising from the term $(n-2)\Delta H$, where ΔH is the equilibrium enthalpy change for the addition of monomer to the prenuclear aggregates. Estimating ΔH as the measured heat of polymerization from our calorimetric and solubility measurements, and using the observed n of 30, the steady state theory predicts an activation energy which is in excellent agreement with the observed value. Dr. Hofrichter's model also explains the unusual time dependence - the initial part of the reaction depends on about the 15th power of the time. He has shown that a novel iterative integration method produces an expression for the rate of nucleus formation that depends on both a high power of the time and concentration.

We have also carried out an extensive thermodynamic investigation using calorimetric and sedimentation methods. A major result is that an excellent first order description of a gel is that it consists of two phases in reversible equilibrium - a liquid phase containing monomers and solvent, and a rigid, much more concentrated phase, containing aligned fibers and solvent. Formation of the fibers is associated with a small positive enthalpy change, indicating that no large conformational change takes place upon gelation. The enthalpy change is itself extremely temperature-dependent, giving a large negative heat capacity change of about -200 cal deg^{-1} mol^{-1}, supporting the view that hydrophobic interactions are important in stabilizing the fiber phase.

Because gelation is like a crystallization process, the thermodynamics and the kinetics are intimately related. The more supersaturated the solution, the faster is the rate of gelation. Using this relationship we are able to predict gelation rates that have not yet been measured from the much larger body of solubility data. These calculations indicate that gelation *in vivo* takes place on times comparable to capillary transit and venous circulation times. Recent experiments by others on cells under physiological conditions confirm these predictions. These results strongly suggest that the rate of gelation may play an extremely important role in determining the course of sickle cell disease. It is in fact possible to correlate a large number of clinical observations with the simple hypothesis that increased clinical severity is associated with a decreased delay time of gelation or increased capillary transit time. The hypothesis suggests that measures which increase the delay time of gelation (with only a minor effect on the solubility) may be of therapeutic benefit. One way of dramatically increasing the delay time would be to decrease the intracellular hemoglobin concentration by only a few per cent. This might be brought about by creating an iron deficiency, thereby "switching" the clinical status from a severe chronic hemolytic anemia with infarctive episodes to a much milder anemia.

Significance to Biomedical Research and the Program of the Institute:

Our kinetic study constitutes a totally new approach to understanding the pathophysiology of sickle cell disease and to the development of therapeutic measures.

Both the theory and methods developed in this investigation should contribute to a general understanding of macromolecular assembly processes.

Proposed Course of Project:

We shall continue our investigation of the kinetics and thermodynamics of hemoglobin S gelation. Dr. Hofrichter has recently developed a unique method to measure both the rate of gelation and the solubility after high speed sedimentation of the fibers on the same sample. With this technique we shall investigate the effect of a wide variety of physiological parameters and possible therapeutic agents on the rate of gelation and solubility of hemoglobin S.

Dr. Hofrichter will independently explore theoretical models for the gelation kinetics which will provide a framework within which to interpret experimental results. The ultimate object of the theoretical study is to develop a basic molecular interpretation of the experimental phenomena. In conjunction with the theoretical studies, he will examine the reaction during the delay time by laser light scattering as a means of following the appearance of small aggregates at times preceeding the onset of bulk gelation. He will further investigate the kinetics for the addition of monomers to the gelled phase by small temperature jumps on preformed gels. Such experiments will permit the observation of the intrinsic temperature dependence of the monomer addition steps, and are primarily aimed at providing basic information necessary for the development of a comprehensive theoretical treatment of the gelation kinetics.

Keyword Descriptors:

Sickle cell disease, hemoglobin S, pathophysiology, calorimetry, macromolecular self assembly, protein polymerization, nucleation theory, sickle cell therapy, blood

Publications:

Hofrichter, J., Ross, P. D. and Eaton, W. A.: Kinetics and mechanism of deoxyhemoglobin S gelation: a new approach to understanding sickle cell disease. Proc. Natl. Acad. Sci. USA 71, 4864-4868, 1974.

Hercules, J. I., Schechter, A. N., Eaton, W. A. and Jackson, R. E., eds., Proceedings of the First National Symposium on Sickle Cell Disease, U.S. Government Printing Office, Washington, D. C., 1974.

Hofrichter, J., Ross, P. D. and Eaton, W. A.: Kinetic and thermodynamic investigation of deoxyhemoglobin S gelation, in the Proceedings of the First National Symposium on Sickle Cell Disease, J. I. Hercules, A. N. Schechter, W. A. Eaton and R. E. Jackson, eds., U.S. Government Printing Office, Washington, D. C., pp. 43-45, 1974.

Ross, P. D., Hofrichter, J. and Eaton, W. A.: Calorimetric and optical characterization of sickle cell hemoglobin gelation.
Hofrichter, J.: Appendix: Theory of the optical birefringence of sickle cell hemoglobin gels. J. Mol. Biol., in press.

Serial No. Z01-AM-29010-03-LCP
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Conformation and electronic structure of biological molecules.

Previous Serial Number: NIAMDD-LCP-12

Principal Investigator: William A. Eaton

Other Investigators: Louise K. Hanson
James Hofrichter

Cooperating Units: Stephen G. Sligar (University of Illinois)

Man Years:

Total: 1
Professional: 1

Objectives:

The general aim of the research is to relate the stereochemistry and electronic structure of biological molecules, particularly proteins with chromophoric prosthetic groups, to their biochemical reactivity and function.

Methods Employed:

In addition to conventional biochemical and spectroscopic methods, techniques have been developed for measuring anisotropic optical properties on microscopic samples. Dr. Hofrichter has designed and constructed a general phase modulation microspectrophotometer which is capable of measuring linear dichroism, circular dichroism, linear birefringence, and circular birefringence with sensitivity comparable to commerical instruments used for macroscopic samples. The instrument also measures direct polarized absorption, and is interfaced to our Honeywell 516 computer.

Major Findings:

An investigation of the optical spectra of the hydroxylating enzyme cytochrome P450 is underway on substrate-bound purified material from the bacterium Pseudomonas putida prepared by Dr. Stephen G. Sligar (University of Illinois). Polarized absorption spectra have been obtained on single crystals of the high spin ferric, high spin ferrous, and low spin carbonmonoxy forms of the enzyme using the microspectrophotometer. The crystal spectra of the high spin ferric derivative reveal intense out-of-plane polarized transitions, which are totally obscured by the in-plane-polarized porphyrin pi electron transitions in the solution spectra. Another unusual feature of the spectra of these complexes is that their near infrared (solution) absorption spectra are unlike those observed for other heme proteins. In order to simultaneously make spectroscopic assignments and elucidate the coordination structure of cytochrome P450 in its various spin and oxidation states Dr. Hanson has carried out extended Hückel molecular orbital calculations on several possible model structures. The results so far suggest that a mercaptide sulfur is one of the axial ligands. The strong interaction of the sulfur orbitals with the iron orbitals may explain the large rhombicity observed in the electron paramagnetic resonance spectra.

b. Electronic Structure of Isolated Hemes:

Investigations are continuing on porphyrin single crystal optical spectra. Free base octaethylporphin (OEP) exhibits a similar out-of-plane polarized spectrum as that previously observed by us in free base tetraphenylporphin. Since OEP contains no aromatic substituents, this finding confirms our assignment of the out-of-plane bands as arising from transitions involving sigma electrons.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the electronic structure of the active sites of proteins should lead to a much deeper understanding of how these molecules perform their biological function.

The techniques developed in our investigations may be applied to a wide variety of problems in protein structure and chemistry.

Proposed Course of Project:

We shall continue our investigation of the electronic structure of hemes using optical spectroscopy and extended Hückel theory. Emphasis will be placed on using anisotropic optical properties of cytochrome P450 to determine its coordination and electronic structure in the various spin and oxidation states. Investigations on porphyrin crystals will be used as model systems for the more complex heme proteins.

Keyword Descriptors:

Heme proteins, cytochrome P450, optical spectroscopy, heme electronic structure, crystal spectroscopy, porphyrins, extended Hückel molecular orbital theory, charge transfer spectra, hemoglobin, redox enzymes

Publications:

Eaton, W. A., Hofrichter, J., Makinen, M. W., Andersen, R. D. and Ludwig, M. L.: Optical spectra and electronic structure of flavine monomucleotide in flavodoxin crystals. *Biochemistry*, in press.

Padlan, E. A., Eaton, W. A. and Yonetani, T.: A crystallographic study of deoxy cobalt(II)mesoprophyrin IX myoglobin. *J. Biol. Chem.*, in press.

Serial No. Z01-AM-29011-04-LCP
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Physics and Chemistry of Photoreception

Previous Serial Number: NIAMDD-LCP-1

Principal Investigator: W. A. Hagins

Other Investigators: W. E. Robinson
S. Yoshikami (Guest Worker)
F. M. Hagins (Guest Worker)
H. Rüppel (Guest Worker)

Man Years:

Total:	5
Professional:	3
Other:	2

Project Description:

The central aim of this project is to describe the chemical and electrical steps that connect absorption of a photon of visible light in a photoreceptor cell with the transmission of a sensory signal across its synapse with the distal neurons of the brain. Previous work by our group on the rod and cone cells of vertebrate retinas have established that an essential part of their excitatory machinery is a large ionic current that circulates through these cells in darkness. This "dark current" enters the plasma membrane covering the light absorbing outer segment of a rod or cone, passes down the cytoplasm of the cell and emerges into the extra-cellular space through the plasma membrane of its more proximal parts. Light absorbed in the outer segment quickly and reversibly reduces the dark current, thus hyperpolarizing the cell and probably changing the rate of release of a neurosensory transmitter substance at its synapse with deeper lying neurons.

During the past year, our work has dealt with three questions. (1) What ionic concentration gradients drive the dark current? (2) How does light control its flow? And (3) What biochemical machinery sustains the dark current and the light-sensitive control mechanism?

Yoshikami and Hagins have approached question (1) by measuring the ionic composition of quick-frozen frog rods directly in an electron microprobe by a new technique that measures electron-induced K_α x-ray emission directly from the frozen tissue @ -150°C without dehydration or fixation. Determination of the Cl^-, Na^+, K^+, and Ca^{++} content of various parts of rod cells support the idea that the dark current consists of a passive influx of Na^+ in the outer segment and a passive efflux of K^+ along the rest of the cell, much like the currents produced by many gland cells and other epithelia. (Hagins, Robinson & Yoshikami, 1975; Hagins & Yoshikami, 1975). Further developments in electron probe microanalysis being conducted jointly with W. C. Lane, Nelson Yew, and Tung Hsu of Etec Corporation, Hayward, California, promise to give information about the role of metabolic inhibitors in controlling the ionic composition of rods and cones as well as other cells that cannot be studied by the usual techniques of cytochemical separation and wet chemical analysis.

The mechanism by which light controls the dark current, (Question 2), has been proposed by us to be one in which Ca^{++} is released from the light-absorbing membranes inside rod outer segments. This Ca^{++} supposedly diffuses to the plasma membrane, where it reacts with Na^+-admitting sites on the inner surface of the membrane, temporarily suppressing their Na^+ permeability and therefore reducing the dark current. Much indirect evidence has been presented in previous reports to support this hypothesis. Three crucial tests of it are to show that (a) raising the cytoplasmic Ca^{++} concentration in rods suppresses the dark current, (b) that internal buffering of the Ca^{++} by metal-binding agents suppresses the effect of light on the dark current and (c) that the internal disk membranes of rods accumulate Ca^{++} in darkness and release it in light. When rods of the living rat retina are treated with the Ca^{++} bearing ionophores, X537A and A23187, externally applied Ca^{++} suppresses the dark current 2000 times more effectively than it normally does. This supports point (a). When live rods are exposed to very low Ca^{++} concentrations for 1 min. or more, they then show a curious change in light sensitivity; the controlling effect of light on the dark current is now enhanced by low levels of added light. This result can be interpreted as indicating that there are Ca^{++} binding sites in rods that can serve as Ca^{++} buffers when depleted of Ca^{++}. These sites would compete with the dark current control sites on the plasma membrane for light-released Ca^{++} in Ca^{++}-depleted retinas. If a low level of light were to release enough Ca^{++} to saturate these buffer sites, however, the sensitivity of the dark current to further light would be enhanced, as is observed. This result supports test (b) of the Ca^{++} hypothesis. No reliable evidence supporting test (c) has yet been found in our laboratory or elsewhere, we believe.

A study of the bioenergetics of photoreceptor excitation has been started. Isolated frog rod outer segments, whose plasma membranes have been shown to be intact by a new staining technique (Yoshikami, Robinson, & Hagins, 1974), have been analyzed for ATP in a continuous stream analyzer using the firefly-tail, luciferin-luciferase system. The cytoplasm of fresh rods contains more than 2 mM ATP. In darkness, about half the ATP disappears in 30-40 min. Sonication of the outer segments causes loss of a substantial fraction of this ATP within 30 sec. If the rods are bleached, the loss on sonication is enhanced by 20 to 60%. The light-induced ATP loss is very rapid and may be caused by rhodopsin phosphorylation, a process observed in several other laboratories. Because of its speed, it is possibly an essential step in visual excitation.

Our studies indicate that retinal rods and cones share much of the electrical and biochemical machinery of neurons. Their most conspicuous difference is their huge dark current, which turns over their entire content of univalent cations in about 1-5 minutes and requires a rate of metabolism as high as that of any known tissue. Light, in controlling this current, not only acts as a sensory stimulus but also must have a great effect on the metabolic load that rods and cones carry. A clear understanding of the metabolism of these cells is therefore important in handling certain retinal vascular diseases and retinal detachment. The scientific importance of understanding the mechanism of visual excitation speaks for itself.

Keyword Descriptors:

Vision, membranes, rhodopsin, ions, photolysis, rods, cones, retina, ATP, phosphorylation

Publications:

Yoshikami, S., Robinson, W. E. and Hagins, W. A.: Topology of the outer segment membranes of retinal rods and cones revealed by a fluorescent probe. Science 185: 1176-1179, 1974.

Hagins, W. A., Robinson, W. E. and Yoshikami, S.: Ionic aspects of excitation in rod outer segments. Ciba Foundation Symposium, 1975. In press.

Hagins, W. A. and Yoshikami, S.: Ionic mechanisms in excitation of photoreceptors. Ann. N.Y. Acad. Sci. In press.

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Hagins, W. A. and Yoshikami, S.: A role for calcium in excitation of retinal rods and cones. Exp. Eye Res. 18: 299-305, 1974.

Serial No. Z01-AM-29012-05-LCP
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The influence of molecular structure on chemical and biological properties

Previous Serial Number: NIAMDD-LCP-3

Principal Investigator: Norman E. Sharpless

Other Investigators: None

Cooperating Units: Frederick S. Brackett, Scientist Emeritus, NIAMDD
William H. Jennings, NIAMDD-LCP

Man Years

Total:	0.5
Professional:	0.5
Other:	0

Project Description:

These projects, in cooperation also with Dr. F. S. Brackett and W. H. Jennings investigates such molecular factors as structure, quantum parameters or physical properties to gain insight into the biological or pharmocological activities of the molecules.

Various forms of spectrophotometry, and other physical, chemical or quantum properties are utilized.

Therapeutic Interference

Therapeutic interference is a negative synergism in which two drugs, each of which is effective, when combined are less effective, or non-effective. We are investigating this phenomenon as evidenced by competitive binding to various biopolymers, and the effect on physical and chemical parameters.

A sensitive probe for structural studies in biopolymers is the technique of spin-labeling. DNA, when so labeled with a nitroxide spin to the extent of 4.8×10^{16} spins/milligram, showed an essentially identical ORD spectrum to the unlabeled DNA, but did show a loss of fine structure in its UV spectrum. There was no discernable difference in binding effects with trypaflavin.

The electron spin resonance of DNA labeled with nitroxide was unchanged with respect to g values or hyperfine coupling constants when reacted with trypaflavin and/or Crystal Violet. However, there is some evidence of signal broadening in the presence of trypaflavin, indicating further loss of mobility of the spin label.

Therapeutic interference is strongly dependent on structures and conformations of the interactants; these are amenable to treatment by a new computer Fortran GEMO. This is a large Fortran program which calculates geometries and corresponding energies of molecules by the strain energy minimization method. Since the biological action of many molecules is a function of their conformations these data are extremely valuable in developing hypotheses on their modes of action. We have obtained a copy of this program from Dr. W. C. Cohen, and are now adapting and expanding it to improve its usefulness to the NIH community. The original program handles only compounds containing carbon, hydrogen and oxygen. For biomedical research purposes, such a program must also be able to treat molecules containing sulfur, nitrogen and phosphorus. Additional parameters involving these elements have been calculated from infrared spectral data. The program should be able to treat molecules containing up to 130 atoms.

One significant addition to GEMO capacity will be display and plotter output routines.

Further Plans

The effects of spin-labeling of biopolymers to obtain information on binding and competition will be continued. Attempts will be made to spin label acridine drugs so as to force the unpaired electron closer to the site of the action. Conformational studies (GEMO) will assist in predicting the feasibility of such syntheses and reactions.

The GEMO program will also be utilized to predict conformations of visual pigments (see report by Adams and Sharpless), and the energetics and conformational states of the vitamin D photoseries, which has remained unresolved for several decades (Brackett).

Keyword Descriptors:

Therapeutic interference, acridine drugs, spin labels, binding studies, biopolymers, energy minimization.

Serial No. Z01-AM-29013-04-LCP
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The physics and chemistry of the visual pigments

Previous Serial Number: NIAMDD-LCP-4

Principal Investigators: Ralph G. Adams
Norman E. Sharpless

Man Years:

Total:	1.5
Professional:	1.5
Other:	0

Project Description:

This project is to elucidate the structure and action of the visual pigment (rhodopsin) chromophore, with the aim of constructing an *in vitro* system behaving similarly to the *in vivo* systems.

Preliminary investigations have shown that the visual pigment chromophore, retinal, can form π-π complexes with either ethanolamine phosphoglyceride (EPG), or phenylalanine, both of which are components of the visual system. Since only three amino acids are known to be intimately associated with retinal after isolation, we have used this fact to postulate that the ultimate chromophore is retinal bound to a three amino acid peptide lysyl-alanyl-phenylalanine, by both an imino bond and a π-π interaction.

Currently we are synthesizing this tripeptide, previously undescribed, and an extremely difficult synthesis, as pointed out previously. The small yields obtained up to now have only permitted preliminary spectral observations which show absorptions at the predicted wavelengths. Comparisons of the optical rotatory dispersion of the tripeptide and of its reaction product with retinal show conformational changes which, while not yet completely interpreted, may be due to helix formation, as suggested by molecular models. Currently the investigations of kinetic and other physical characteristics are being delayed due to the difficulty inherent in the synthesis of the tripeptide. However, we have initiated a contract with Miles Laboratories to furnish us with what we hope to be a pure sample in adequate amounts.

Literature observations indicate that some of the spectral characteristics of the π-π complex may be the result of acetal formation caused by the methanol in the chloroform-methanol system used as a solvent. Tri-t-butyl carbinol, a molecule in which the hydroxyl group is hindered has been shown to be extremely unreactive towards alcohol reagents and therefore not capable of forming acetals. When substituted for methanol in the alcohol-chloroform solvent system, no acetal could be detected. Conversely, an imine was formed when EPG was added but at a extremely diminished rate. Whether the acetal is critical as an intermediate in imine formation is equivocal from present data.

Further investigations into the carbinol as an interesting molecule in itself indicate by NMR temperature studies that its methyl groups become increasingly less equivalent as evidenced by splitting of the NMR band. Mass spectra also show that instead of forming a molecular ion, the compound preferentially loses a molecule of ethylene from the extremely crowded methyl groups.

Our current computer programming for conformational studies is inadequate but we have high hopes for the new program now being modified for biological molecules. This should allow us to compute the most probable conformations for comparisons with observed properties.

Keywords:

Vision, retinal, rhodopsin, tripeptides, π-π complexes, acetals-tri-t-butyl carbinol, phospholipids, ORD.

Serial No. Z01-AM-29014-15-LCP
1. Chemical Physics
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of altitude (hypoxia), exercise, and other environmental stresses on physiological, biochemical and pathological mechanisms in animals.

Previous Serial Number: NIAMDD-LCP-10

Principal Investigator: Paul D. Altland

Other Investigators: H. F. Brubach
M. G. Parker

Cooperating Units: Barnett Rattner (Pre-doctoral Guest Worker)
Department of Zoology, University of Maryland

Man Years:

Total:	3.5
Professional:	2.5
Other:	1.0

Project Description:

To determine physiological, biochemical and pathological changes which occur following exposure to altitude (hypoxia) and exercise in the cold. To determine the effect of hypoxia and age on the susceptibility of rats to experimental bacterial endocarditis. Altitude acclimatization was carried out in large decompression chamber. Exercise was performed in a revolving drum equipped for applying exercise of varying degrees of severity. Experiments were conducted in walk-in cold room at 5°C.

The hypothesis that long term exposure to one adverse environment may lead to increased or decreased tolerance to other adverse environments not previously experienced has been advanced by a number of investigators including Sundstroem, Selye, Zimkin and Barbashova. Little is known of the effects of multiple-agent environments. For example, no studies have been reported in which altitude-acclimatized animals were simultaneously exposed for the first time to exercise and cold at ground level. It has been found that exercise tolerance of rats acclimatized for 6 weeks to 18,000 ft. simulated altitude was lower than in unacclimatized rats during 9 hours exercise (6.9 meters/min.) in the cold (5°C). The reduced exercise tolerance was attributed to severe hypothermia with associated decreased metabolism, severe polycythemia (hematocrit 69), hypoglycemia (blood glucose 80 mg/100 ml), lower liver glycogen, lower serum lactic acid concentrations and a higher incidence of myocardial necrosis and earlier development of striated muscle necrosis. No such changes occurred in altitude (18,000 ft.) acclimatized rats exercised at room temperature (23°C). These findings supports the hypothesis that available homeostatic mechanisms become exhausted more readily in multiply stressed than in singly stressed rats. It also provides evidence of a negative cross-adaptation between altitude and other stresses such as cold and exercise.

Experiments are being conducted on the responses (physiological and biochemical) of rats subjected passively to an environment containing cigarette smoke while at rest and during exercise of varying degrees of severity. A chamber is being developed in which rats may be subjected varying concentrations of smoke and carbon monoxide. Study will be made of the independent effect of nicotine on resting and exercising rats.

Studies are also being conducted on the relationship of polycythemia and serum hemoglobin on hyperbilirubinemia in rats exposed to 18,000 ft. simulated altitude.

Keyword Descriptors:

Altitude acclimatization, exercise tolerance in cold, multiple stresses, cross-adaptation, hypothermia, polycythemia, myocardial necrosis.

Publications:

Altland, P. D.: Tolerance of altitude acclimatized rats to exercise in the cold. *Proc. Soc. Exp. Biol. Med.* (in press).

REPORTED IN PRESS - 1974

Altland, P. D., Highman, B., and Sellner, R. G. (with the technical assistance of M. G. Parker): Serum enzyme and tissue changes in shaven rabbits exposed to cold. *Cryobiol.* 11: 296-304, 1974.

Serial No. Z01-AM-29015-04-LCP
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Digital computer facilities for LCP and LMB

Previous Serial Number: NIAMDD-LCP-2

Principal Investigator: William H. Jennings, Jr.

Other Investigators: None

Cooperating Units: Computer Systems Laboratory, DCRT

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

Development of a multi-user laboratory computer facility for real-time data acquisition and processing.

Major Findings:

This facility was in routine operation this year without serious problems with either hardware or software. Only a few changes have been made to the system: (1) a direct data access device and two telephone lines have been installed to permit higher data rates between the Tektronix 4012 graphics terminal and the DCRT PDP-10 machine. (2) The EPR (electron paramagnetic resonance) interface, anticipated in last year's report, has been installed and the software is presently under test. (3) The operating system, OLERT, was upgraded to Revision E and a number of Fortran programs were revised in order to take advantage of the new OS features. This upgrade, which was immediately followed by the departure of the system programmer, proved an opportune time to catalog and index all programs, tapes, cards, manuals and listings. Much of the system documentation is now in excellent condition.

The programming development on this system during the year was divided between several dozen small, special purpose processing programs and three very large general use programs. The latter are: a disc based editor, a multi-option table processing and display program and a versatile Cal Comp plotting program. These three are structured programs descended from existing system programs. They are essentially open-ended; therefore, development will continue as new needs arise.

In another area, programs have been developed to transfer standard data tables (via magnetic tapes) to the DCRT PDP-10 disc. There the tables may be used as data bases for the sophistocated programs available on that machine.

Significance to Biomedical Research and the Program of the Institute:

This facility has become an integral element in the research projects of many investigators in LMB and LCP. The ability to quickly acquire and process large quantities of data, particularly from short-lived chemical or biological preparations, permits important experiments that would normally be considered so tedious as to be marginally useful.

Proposed Course of Project:

Program development will continue on the large, general use programs and on utilities to transfer data tables to larger machines. Heavy use of the data acquisition programs has so reduced the capacity of the system to service data processing needs that alternative methods will be implemented. These include structured programs with small functional segments which may also be used as independent programs and programs on the DCRT PDP-10 which emulate existing processing programs.

Equipment changes are contemplated for FY76 or FY77 but the review of the system conducted in FY75 showed that piece by piece replacement of specific computer components is usually impractical. We are currently reevaluating the System Definition of 1967 and expect to develop a revised System Design for staged implementation over the next five years.

Keyword Descriptors:

Laboratory computer, hierarchical computers

Publications:

Jennings, W. H.: A minicomputer facility in the research laboratory: A case history and a look forward. Proceedings: Minisystems '75, April 30 - May 1, 1975, Honeywell Information Systems, Boston, Mass. pp. 355-371.

ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

The Laboratory of Physical Biology is carrying out studies in a broad range of research areas.

Molecular Structure

Immunoglobulin. Information about the structure of biologically important protein macromolecules can be obtained by high resolution electron microscopy. The size and shape of these molecules can be determined most accurately when they are crystallized into three-dimensional structures. Protein crystals that are large enough can be studied by X-ray diffraction techniques, with such a study leading, under favorable conditions, to a complete three-dimensional placement of the amino acids in the protein molecule. Although the electron microscope can not give comparable detailed information, it can give the rough size and overall shape of the molecule, and its approximate location in the crystal lattice. This information is helpful at the beginning of a detailed X-ray analysis. The electron microscope can also give this type of information for protein crystals too small for X-ray studies, and for crystals that are not sufficiently stable under X-ray bombardment to make a detailed X-ray analysis feasible.

An orthorhombic crystal of Fab' fragments of a mouse myeloma immunoglobulin protein has been examined in the electron microscope. Projections in the three axial directions gave clues about the positions of the molecules in the crystal. These were useful for the low resolution X-ray analysis. A protein crystal in an insect fat-body was also analyzed from electron micrographs. The protein was shown to be approximately spherical, 132 Å in diameter, with a core about 60 Å in diameter containing an electron-dense material. If this protein is caliphorin, this core probably contains calcium. (Labaw)

Muscle. A high spatial resolution, position sensitive electronic detector for X-rays has been developed in this laboratory. The quality of the recordings is considerably superior to those made with photographic film and, at this stage, the speed is at least 10 times faster. (St. Onge)

Using this detector, equatorial diffraction patterns with several high order reflections have been consistently obtained from resting muscles. A reflection not indexible on the hexagonal lattice of the myofilaments has been studied. The spacing of this reflection is found to vary proportionally with those originating from the hexagonal lattice. Although the origin of the non-indexible reflection is not known, its spacing is close to that seen in electron micrographs of the Z disc, which raises the possibility that the Z disc is a significant source of compliance in activated muscle fibers. (Yu, Lymn, Podolsky)

The interpretation of X-ray diffraction patterns is not completely straightforward. In particular, the equatorial reflections from muscle provide information in only two dimensions. Hitherto, only the two principal reflections have been studied in detail, and they have been interpreted in a very plausible picture of radial movements by projections from the thick myosin-containing filaments. However, there are other reflections, which are particularly visible using the new camera-counter system, and a study was undertaken to discover how much information could be obtained from the additional data.

Using the data directly, one can mathematically synthesize the possible structures which might give rise to the diffraction patterns seen. The detail seen in these syntheses increases with N, the number of reflections included in the calculations. But, for muscle equatorial diagrams, the number of possible syntheses increases as 2^N. (The maximum N we have observed is seven). However, much is known about the gross molecular structure of muscle and use of this knowledge makes it possible to rule out many of the possible syntheses.

In collaboration with Dr. G. Cohen (LMB), a computer was used to predict the X-ray diffraction pattern from many models of molecular packing in muscle. Analysis of these results leads to certain general statements about the relation between packing and diffraction pattern. When these statements, and the knowledge of the gross structure, are applied to the possible reconstructions based on seven reflections, it was found that, in the relaxed state, (a) the myosin projections extend to the radius of the actin filament, and (b) the rotational symmetry around the fiber axis is either 6- or 9-fold. (Lymn)

Red cell sickling. Sickle cell anemia is one of the few hereditary diseases of man in which the disease process at the molecular level is understood; the defective molecule has not only been identified as a distinctive hemoglobin (HbS), but the abnormal interactions of this molecule can be explained. Work on HbS has advanced both in vivo and in vitro. With regard to the chemistry of HbS, it was found that aggregation as well as solubility can be inhibited by inorganic ions according to the classical Hofmeister series. Observations reported from many laboratories are consistent with an aggregation mechanism due to hydrophobic interactions by deoxy HbS, as postulated by Murayama, and are a consequence of the β^6 glu → val substitution. Furthermore, a series of polyhydric alcohols were investigated with Dr. S. Shifrin and C. L. Parrott (NCI); the effectiveness of deoxy HbS to aggregate increases with the number of hydroxymethyl groups. In terms of oral urea therapy, more data were collected regarding the effectiveness of urea in ameliorating the symptoms of sickle cell crises. These cases were studied in Ghana, in collaboration with Dr. Oku Ampofo. (Murayama, Levine)

Cell Membranes

Lipid monolayers and bilayers. Studies of the phase relations for mixtures of lecithin and cholesterol indicate that complex formation occurs in water with a stoichiometry of 2 moles lecithin to 1 mole cholesterol. Thus for compositions in which lecithin exceeds this ratio, two phases exist--lecithin and the complex. A method for isolating the complex has been developed. The physical state of the complex is relatively insensitive to temperature between 0° and 40°C. However, the physical state of the excess lecithin may undergo several different phase changes at temperatures which depend on the particular lecithin used. For example, dimyristoryl lecithin undergoes a gel ⇄ liquid-crystalline transition at about 24°C; at 30°C the particles spontaneously form a highly dispersed state with particle sizes in the range of 100-1000 Å. In this size range the surface properties of the lipid particles alter the lecithin-cholesterol phase relations. The implications of this finding with respect to phase transitions in membranes is under study. (Gershfeld)

The analysis of the role of lipids in determining the electrical properties of the hyphal membrane of the mold Neurospora crassa has been continued using standard microelectrode techniques. These studies involve comparing the wild type N. crassa with a mutant where the fatty acid composition of the mutant membrane may be varied by alterations in the growth medium. In our studies the amount of plamitate in the mutant membrane was doubled over the amount in the wild type membrane. The major findings this past year include:

(a) a gel-liquid crystal phase transition occurs at -20°C for wild type and at -10°C for the palmitate mutant cell. A second phase transition is detected at 33°C in both N. crassa strains. These findings have been observed with extracts of membrane lipids as well as intact Neurospora hyphae, using differential scanning calorimetry.

(b) the temperature dependence of membrane resistance (R_m) for the palmitate mutant is continuous between 7° and 40°C. In contrast, for the wild type a dramatic decrease in R_m occurs between 8° and 20°C; the temperature dependence below and above this interval is similar to that for the palmitate mutant. Thus while a change in the palmitate composition of the membranes does alter the phase relations in the respective membranes, the discontinuity in the R_m-temperature relation for the wild type cells does not occur in interval where any obvious lipid phase changes occur. This suggests that membrane lipids may have only an indirect effect on ion permeability. (Friedman, Gershfeld)

Red cell membrane. Cholesterol, one of the major lipid constituents of mammalian cells, is known to have significant effects on the physical properties of artificial lipid membranes. Erythrocytes are particularly well suited for studies on the function of cholesterol in cell membranes, since it is possible to remove up to 40% of their cholesterol by extraction with mildly modified plasma, a treatment which seemingly does not cause extraneous changes in the cell membrane. A number of studies have shown that removal of this amount of cholesterol has only minor effects on the physical and permeability properties of erythrocyte membranes. Work of the past year was directed towards increasing the cholesterol depletion beyond the 40% level. Despite exploration of a wide range of experimental conditions, this depletion level could not be exceeded. Experiments with "inside-out" membrane vesicles, in which the normally cytoplasmic surface of the membrane is in contact with the plasma, established that the limitation on the cholesterol depletion is not a result of the remaining cholesterol being located at the inner surface of the membrane where it is inaccessible to plasma. It thus appears that there are two classes of cholesterol in the erythrocyte membrane, one readily removable and the other firmly bound. However, as yet, the possibility can not be excluded that the limitation is a result of a structural change in the membrane that occurs after the first 40% of the cholesterol has been removed. (Gottlieb)

The cross-bridge mechanism. Several new methods for studying the cross-bridge mechanism have been exploited further. One makes use of a method for determining stiffness by measuring the propagation speed of small mechanical disturbances. In muscle, this is also a measure of the interaction between the thick and thin filaments. The technique was applied to the tendon of a muscle. The tendon was found to behave as an exponential compliance, that is, the stiffness of the tendon was proportional to the force load upon it. The technique was also applied to skeletal muscle under different loads, and it appeared as if the instantaneous stiffness of muscle was also a function of load. Experiments are being set up to determine the relative contribution of the muscle sarcomere and the tendon to the overall stiffness change. (Schoenberg)

The second new method makes use of our ability to measure the contraction transients of skinned muscle fibers. The effects of ionic strength, pCa and ATP on the isotonic transients of frog skinned fibers have been examined in greater detail. Within the resolution limits of the present technique, it is confirmed that calcium switches "on" the cross-bridges. Ionic strength changes the transients and acts by modifying the kinetic parameters in a cross-bridge cycle.

In order to improve the resolution with which the transients are analyzed, a procedure was developed for chemically "skinning" a mammalian (the rabbit psoas) muscle in small bundles. Both the steady and the transient motions are greatly slowed in the rabbit psoas fibers. Many of the earlier findings on the frog have been confirmed on the rabbit. The ionic strength affects the pre-steady motion but not the steady speeds, confirming the earlier conclusions on frog fibers. The results with rabbit show clearly that lowering the ionic strength suppresses the amplitude of the transient. The slow phase of the transient is accelerated in low KCl. The influence of KCl on the quick displacement and/or the rapid phase of the transient still cannot be resolved. (Gulati)

The activation mechanism. A method has been developed for measuring simultaneously the isometric force and ^{45}Ca efflux from a single skinned frog fiber segment over several successive time intervals. After ^{45}Ca release from the sarcoplasmic reticulum has been induced by application of Cl ion ("depolarization"), the direction of subsequent ^{45}Ca movement was altered by increased magnesium levels. The results showed directly for the first time that magnesium ion promotes Ca reaccumulstion in situ. (Stephenson)

Cardiac muscle. In sheep cardiac Purkinje fibers, the membrane conductance and capacitance per apparent surface area were found to increase with diameter. This implies that much of the membrane contributing to the passive electrical properties is not on the surface but within the volume of the fiber. Since the time constant for charging this membrane is about 2 msec, the non-surface membrane could very well be an important factor in excitation. Presently, the "active" currents generated by such membranes are being explored to see if they can contribute significantly to the generation of the fiber action potential. (Schoenberg)

Cellular organelles. Euglena and Neurospora have been shown to possess a high degree of internal organization. There are sub-microscopic enzyme complexes which catalyze many sequential reactions. Even the simplest descriptions of physical binding of enzymes and substrates leads to testable predictions about organized metabolism. Biochemical systems are known in Neurospora and yeast which fit these models. Organized pathways in Euglena are suggested by the rigid control of the amino acid pool and by evidence for amino acid biosynthesis via bound intermediates. The amino acid composition of cellular protein is a product of this control and still further regulation.

Vitamin B12 is needed by man to prevent prenicious anemia and by Euglena for cell growth. The biochemical activity of the vitamin is unknown in the protozoan, but present experiments suggest that vitamin B12 starvation leads to a blockage at a late stage in the cellular division process. (Shehata, Kempner)

Insect metamorphosis. Metamorphosis in the blowfly entails an extensive destruction of the larval cells concomitantly with the differentiation and growth of the structures that will give rise to the adult insect. The entire process takes place within the span of a week. The question arises as to what fraction, if any, of the larval structures and/or organelles is preserved during the course of this process. A case study is the fate of ribosomal RNA.

It was found that the pool of ribosomal RNA remains essentially constant (average 200 μg) during the metamorphosis. There is a low value (170 μg) during the early pupal stage and a high value (230 μg) during the late pupal stage. The pool of ribosomal RNA was found to turn over with a half life of 8.5 days. This value corresponds to a rate of synthesis of 36 μg of rRNA/day/insect. If the specific activity of the precursor pools is considered, a rate of synthesis of 36 μg of rRNA/day/insect may be estimated.

The pools of 18S and 28S ribosomal RNA turn over with statistically indistinguishable half lives, thus suggesting similar degradation rates for both ribosomal subunits.

Injection of radioisotopes into blowfly pupae allowed direct measurement of the rates of ribosomal RNA synthesis during metamorphosis. The rates of synthesis thus obtained constitute an upper limit to the rate of synthesis of rRNA during metamorphosis and are from 2 to 4.8 times higher than the values obtained from turnover studies. These higher values may also indicate that the UTP pool which provides the immediate precursors for RNA synthesis behaves as a small compartment in rapid equilibrium with externally supplied nucleotides, as has been found to be the case for mammalian cells in culture.

The rates of synthesis of ribosomal RNA obtained both through turnover studies and by direct measurement indicate that at least 50% and possibly most of the ribosomes in the adult blowfly have been synthesized de novo during metamorphosis. Thus there is little or no carry-over of ribosomes from the larval to the adult stage.

Finally, turnover studies have shown that the pyrimidine nucleotides in RNA turn over with different half lives. (Protzel)

Biological Rhythms

The statistical model developed to discriminate timer and effector elements in the endogenous pacemaking of human finger-tapping continues to prove valuable in other contexts. Analysis of data on circadian eclosion (the emergence of an adult fly from the pupal case) rhythms in *Drosophila*, for example, has shown that it is not temperature-independent, as originally thought, but temperature-compensated. Applied to cell division cycles the supposedly sequentially triggered cell division and DNA synthesis periods can be shown to be not necessarily in a cause-and-effect relationship but under control of a timer which can schedule each aspect of the cycle separately. (Mets)

Project No. Z01 AM 27000-13 LPB
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Mechanism of Muscular Contraction

Previous Serial Number: NIAMDD-LPB-1

Principal Investigator: R. J. Podolsky

Other Investigators: Richard W. Lymn
Elizabeth W. Stephenson
Leepo C. Yu
Jagdish Gulati

Cooperating Units: Dr. Richard St. Onge, Department of Physics, University of New Hampshire

Man Years:

Total:	7
Professional:	5
Other:	2

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. The X-ray diffraction patterns of muscle cells in different physiological states were examined with a high resolution, position sensitive, electronic detector. In resting cells, many orders of diffraction were observed from the hexagonal filament lattice. The relative intensities of these reflections indicate that the myosin projections extend almost to the radius of the actin filament, and that during the activation the motion of these projections is more azimuthal than radial.

2. A reflection not indexible on the hexagonal lattice of the myofilaments was characterized. The spacing of this reflection (the α reflection) corresponds to that seen in electron micrographs of the Z disc. When sarcomere length was changed, the α reflection spacing varied proportionally with the hexagonal lattice reflection, which suggests that the Z disc may be a source of compliance in activated muscle fibers.

3. The diffraction pattern of actively shortening muscle (low force) is similar to that of muscle contracting isometrically (high force). The diffraction pattern of the low force state is not associated with a transition toward the "resting" diffraction pattern.

4. A procedure was developed for chemically "skinning" a mammalian (the rabbit psoas) muscle in small bundles. The physiological properties of these fibers are similar to those of frog fibers, but the motion at a given temperature is much slower.

5. A method was developed for measuring simultaneously the isometric force and ^{45}Ca efflux from a single skinned frog fiber segment over several successive time intervals. It was shown directly that the modulating action of magnesium ion on the mechanical response is due to its promotion of calcium reaccumulation by the sarcoplasmic reticulum.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.

2. The influence of these same agents on myofilament spacing and the cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The effect of motion on the number of cross bridges in intact, electrically-stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Keyword Descriptors:

muscle contraction, muscle activation, X-ray diffraction, Z line filament lattice, skinned muscle fibers, position sensitive detector

Publications:

Podolsky, R. J.: Muscle activation: The current status. Fed. Proc. 34: 1374-1378, 1975.

Hill, Terrell L., Eisenberg, Evan, Chen, Yi-der, and Podolsky, R. J.: Some self-consistent two-state sliding filament models of muscle contraction. Biophys. J. 15: 335-372, 1975.

Podolsky, R. J.: Cross-bridge properties derived from muscle contraction transients. In Proc. IV Int. Biophys. Congr. [1972]. Pushchino, USSR, Academy of Sciences of the USSR, 1973, Vol. 1, pp. 438-446.

REPORTED IN PRESS - 1974

Schoenberg, M., Wells, J. B. and Podolsky, R. J.: Muscle compliance and the longitudinal transmission of mechanical impulses. J. Gen. Physiol. 64: 623-642, 1974.

Project No. Z01 AM 27001-01 LPB
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Contractility and Excitability of Skeletal and Cardiac Muscle

Previous Serial Number: None

Principal Investigator: Mark Schoenberg

Other Investigators: None

Cooperating Units: Dr. Jay B. Wells, Medical Neurology Branch, NINCDS

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Goals:

1) To understand the molecular and structural basis of muscular contraction.

2) To determine the factors controlling the excitability of heart muscle.

Methods and Major Findings, Goal 1:

1) A method has been developed for determining stiffness by measuring the propagation speed of small mechanical disturbances.

2) This technique has been applied to the tendon of a muscle. The tendon was found to behave as an exponential compliance, that is, the stiffness of the tendon was proportional to the force load upon it.

3) The technique was also applied to skeletal muscle under different loads. It appeared as if the instantaneous stiffness of muscle was also a function of load.

4) Experiments are being set up to determine the relative contribution of the muscle sarcomere and the tendon to the overall stiffness change with load.

Methods and Major Findings, Goal 2:

1) In sheep cardiac Purkinje fibers, the membrane conductance and capacitance per apparent surface area increases with diameter. This implies that much of the membrane contributing to the passive electrical properties is not on the surface but within the volume of the fiber.

2) The time constant for charging this membrane is about 2 ms. This indicates that the "non-surface" membrane could very well be an important factor in excitation.

3) The velocity of the Purkinje fiber action potential conduction is relatively independent of diameter. This implies, that although the internal membranes may be important for excitation, they are not important for conduction.

Significance to Biomedical Research and the Program of the Institute:

The experiments on the mechanism of muscular contraction are significant for two reasons. First they contribute to a sound understanding of how normal muscle functions (which is vital to any study of diseased muscle) and secondly, the same type of structure responsible for muscle contraction appears to be common to many other cells in which motility plays a role such as platelets, nerve axons, and many others.

The study of heart excitability is of course of great interest in that a fairly large fraction of deaths from myocardial infarction are related not to the physical destruction of large amounts of tissue, but rather to small injuries which result in destruction of normal electrical activity. One question our work has shed light on is the question of how the geometry of Purkinje fibers might be important in coordinating electrical activity. Another is what factors are necessary to describe the state of excitability of heart muscle (and other cells as well) and also how might these factors be influenced by other parameters in the system such as potassium concentration, drugs, etc.

Future Course:

Goal 1

1) Additional experiments will be undertaken to differentiate between compliance in the tendon and that in the sarcomere. Knowledge of compliance at the sarcomere level is important in determining the rate constants for cross-bridge formation as well as the mechanism of force generation.

Goal 2

1) The study of the internal membranes will be extended from the purely passive membrane to the active membrane in order to further delineate the importance of these membranes for fiber excitation.

Keyword Descriptors:

muscle contraction, electrical excitability, stiffness of muscle, cardiac electrophysiology, muscle cross-bridge kinetics, Purkinje fibers, frog semitendinosus muscle, microelectrodes, cardiac arrythmias

Publications:

Schoenberg, M., Wells, J. B. and Podolsky, R. J.: Muscle compliance and the longitudinal transmission of mechanical impulses. J. Gen. Physiol. 64: 623-642, 1974.

Schoenberg, M., Dominguez, G. and Fozzard, H. A.: Effect of diameter on membrane capacity and conductance of sheep cardiac Purkinje fibers. J. Gen. Physiol. 65: 441-458, 1975.

Project No. Z01 AM 27002-12 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Sense of Rhythm.

Previous Serial Number: NIAMDD-LPB-2

Principal Investigator: John B. Buck

Other Investigators: Dr. Laurens Mets and Mr. Charles H. Hanna

Cooperating Units: Mrs. Elisabeth M. Buck, Guest Worker; Dr. Frank A. Hanson, University of Maryland, UMBC, Catonsville, Maryland; Dr. James F. Case, University of California, Santa Barbara, California.

Man Years:

Total:	3.5
Professional:	2.5
Other:	1.0

Project Description:

Objectives:

To understand the mechanism and function of certain rhythmic motor behavior in selected higher organisms and rhythmic cellular processes in selected microorganisms. To understand how oscillatory activity in one organism can be entrained to that in another.

Methods Employed:

Standard electrophysiological, microbiological and psychophysical. Special computer analyses.

Major Findings:

1. Pacemaker behavior in an as-yet unidentified Melanesian lampyrid was analyzed in relation to phasing of artificial input signals. In this organism the pacemaker is completely reset in one cycle, as previously reported for *Pteroptyx cribellata*, but in addition transient effects amounting to more than 10% of the endogenous period and damping out only by the 6th post-signal cycle, were measured. (J. Buck; E. Buck).

2. A model for the *Pteroptyx cribellata* pacemaker was developed on the basis of certain pacemaker neurons in *Aplysia*.

3. An unexpected diversion from the physiology of flash control was provided by a paper (J. E. Lloyd, 1973) criticizing an old theory of ours about the significance of mass synchronous flashing (Buck and Buck, 1966). A manuscript has been prepared in which new information from field studies has been marshalled and Lloyd's and other hypotheses critically examined. The upshot is to indicate that the mass synchronization is a true group-benefit effect. On the scale we observed, the phenomenon is unprecedented. (J. Buck and E. Buck).

4. The model developed for distinguishing properties of the endogenous pacemaker from those of the motor effector process in human fingertapping rhythms has proven applicable to a wide range of rhythmic biological phenomena and has led to significant new insights. Analysis of literature data on circadian rhythms in *Drosophila* eclosion indicate that although the period of the rhythm is relatively temperature independent, its precision is strongly temperature dependent. From this simple consideration, it is clear that the mechanism of the "circadian clock" is not temperature independent, but rather temperature compensated. With this method of analysis, it is possible to demonstrate the independence of a circadian rhythm from environmental cues, even when its average period is exactly 24 hr. Persistent synchrony of cell division in *Euglena* populations was previously thought to require intercellular communication and mutual synchronization, though experiments designed to test this hypothesis have all proven negative. The present model of the clock mechanism explains the persistence of synchrony without requiring intercellular communication. (L. Mets).

5. Analysis of cell division cycles with the methods developed for fingertapping rhythms yields a model which is fundamentally different from that implicit in the traditional G_1-S-G_2-M analysis. The cell cycle is viewed as regulated by a "scheduling process" which determines the timing of events. Each aspect of the cell cycle (e.g. DNA synthesis, mitosis, division, etc.) may be separately scheduled to occur at a specific time. Thus, the order of events in the cell cycle does not necessarily define cause and effect relationships. Some of the implications of this model are currently being tested in studies of the cell cycle in the green alga, *Chlamydomonas reinhardi*. (L. Mets).

6. Work has continued on the identification of lampyrid photocyte granules with microbodies. (C. Hanna).

Significance to NIAMDD Research:

The new data on rhythms and entrainment encourage the hope of eventually defining human pacemaking activity, separating timing from motor steps and understanding the ability to entrain to external pacing. Analysis of cellular and subcellular physiological rhythms is an increasing objective.

Keyword Descriptors:

Rhythm
Synchrony
Pacemaker
Circadian Rhythm
Microorganism
Cell Division
Evolution
Euglena
Chlamydomonas
Insect

Proposed Course of Project:

No major changes anticipated.

Project No. Z01 AM 27003-06 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Dynamic Properties of Cell Membranes and Related Systems

Previous Serial Number: NIAMDD-LPB-3

Principal Investigator: N. L. Gershfeld

Other Investigators: K. J. Friedman
K. Tajima
J. N. Weinstein

Cooperating Units: S. Razin, Department of Clinical Microbiology, Hadassah Medical School, Jerusalem, Israel

Man Years:

Total:	4.5
Professional:	3.5
Other:	1

Project Description:

The objective of this project is to establish a physico-chemical basis for the dynamic properties of cell membranes. Among these properties and the questions of particular interest are the mechanism of cell membrane assembly, membrane permeability, and membrane mediated morphological transformation. The research to date has focused on the role which lipids fulfill in these processes. This has largely been examined by complementary studies of the physical properties of lipids (thermodynamics of multicomponent mixtures in various states of aggregation) and of the membrane properties of a variety of cellular systems (sciatic nerve of *Bufo marinus*, sea urchin eggs, mycoplasma, and the mold *Neurospora crassa*).

The systematic study of the physical properties of lipids and lipid suspensions in water has resulted in the formulation of a set of rules for predicting lipid miscibility in complex mixtures of bulk, monolayer, and lamellar bilayer systems. Each of these systems is dominated by the contribution of the hydrocarbon moiety of the molecules to the internal energy of the lipid aggregate. Methods for obtaining the internal energies of condensed lipid systems have been reported previously.

Major Findings:

1. Physico-chemical properties of lipid mixtures

(a) A spontaneous transformation of lecithin in water from the liquid-crystalline state to a highly dispersed state at a unique temperature has been observed; preliminary evidence suggests that the new state is composed of single bilayers (Tajima).

(b) Lecithin and cholesterol form stable complexes with a stoichiometry of 2 moles lecithin to 1 mole cholesterol. A method for isolating the complex has been developed.

2. Membrane lipids of Neurospora crassa and the electrophysiological properties of the hyphal membrane (Friedman)

These studies involve comparing the wild type N. crassa with a mutant where the fatty acid composition of the mutant membrane may be varied by alterations in the growth medium. In our studies the amount of palmitate in the mutant membrane was doubled over the amount in the wild type membrane. The major findings this past year include:

(a) a gel-liquid crystal phase transition occurs at -20°C for wild type and at -10°C for the palmitate mutant cell. A second phase transition is detected at 33°C in both N. crassa strains. These findings have been observed with extracts of membrane lipids as well as intact Neurospora hyphae using differential scanning calorimetry.

(b) the temperature dependence of membrane resistance (R_m) for the palmitate mutant is continuous between 7° and 40°C. In contrast for the wild type a dramatic decrease in R_m occurs between 8° and 20°C, while the temperature dependence below and above this interval is similar to that for the palmitate mutant. Thus while a change in the palmitate composition of the membranes does alter the phase relations in the respective membranes, the discontinuity in the R_m-temperature relation for the wild type cells does not occur in an interval where any obvious lipid phase changes occur. This suggests that membrane lipids may have only an indirect effect on ion permeability.

Future Course:

(a) The structure of the lecithin-cholesterol complex will be examined using electron microscopy and X-ray diffraction.

(b) Spontaneous lecithin transformations in suspension will be studied with laser light scattering techniques.

(c) The question of membrane lipid phase transitions and their influence on the electrical properties of the membranes will continue with the use of additional mutants.

Keyword Descriptors:

cell membranes, membrane permeability, thermodynamics, mycoplasma Neurospora crassa, monolayer, lecithin, cholesterol, differential scanning calorimetry

Publications:

Tajima, K. and Gershfeld, N. L.: Thermodynamics of monolayer solutions of lecithin and cholesterol mixtures by the surface vapor pressure method. Advances in Chemistry Series, in press.

Reported in Press - 1974

Gershfeld, N. L., Wormser, M. and Razin, S.: Cholesterol in mycoplasma membranes. I. Kinetics and equilibrium studies of cholesterol uptake by the cell membrane of Acholeplasma laidlawii. Biochim. Biophys. Acta 352: 371-384, 1974.

Razin, S., Wormser, M. and Gershfeld, N. L.: Cholesterol in mycoplasma membranes. II. Components of A. laidlawii cell membranes responsible for cholesterol binding. Biochim. Biophys. Acta 352: 385-396, 1974.

Project No. Z01 AM 27004-05 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Physical Chemistry

Previous Serial Number: NIAMDD-LPB-4

Principal Investigator: Melvin H. Gottlieb

Other Investigators: None

Cooperating Units: Dr. E. D. Eanes, LBS/NIDR

Man Years:

Total:	1.67
Professional:	1
Other:	0.67

Project Description:

The overall objective is to determine the role of the various lipid components of cell membranes in maintaining membrane structural and permeability properties. Present work centers on cholesterol in erythrocyte membranes, the approach being the comparison of physical properties (i.e. X-ray diffraction, spectroscopic, calorimetric) of normal membranes with those from which cholesterol has been removed. This approach necessarily requires that the cholesterol removal be accomplished with a minimum of extraneous modification of the membranes. Some years ago other workers reported a procedure which seemingly meets this requirement and has since been used in several studies. Unfortunately no more than about 40% of the cholesterol is removed by the described procedure, and it has not been possible to study the properties of erythrocyte membranes completely devoid of this steroid. During this past year we have attempted to determine the reasons for the limitation to cholesterol removal by the described procedure, and to attain greater depletions by modifying the procedure.

The method in question involves incubation of erythrocytes in plasma that has been "preincubated" to convert a portion of its lipoprotein cholesterol to cholesterol ester. The depletion of cholesterol from the erythrocytes occurs as a result of its transfer to the (unesterified) cholesterol-poor plasma lipoproteins. We have established that the limitation on cholesterol depletion by this procedure is not a result of an insufficiency of the unesterified cholesterol-poor lipoprotein "sink" for the erythrocyte cholesterol, of inadequate time for equilibration of cholesterol between the erythrocytes and the plasma lipoproteins, nor in some way due to the lysolecithin, which is produced in large amounts in the plasma during the incubation step. Dialysis of the plasma, changing plasma pH between 6.0 and 9.0, addition of albumin, hemolysis of the cells prior to incubation, and replacing the preincubated plasma by fresh preincubated plasma during the incubation, all had no effect on the cholesterol depletion. Experiments with "inside out" erythrocyte membrane vesicles, in which the normally cytoplasmic surface of the membrane is exposed to the plasma and *vice versa*, established that the limitation on cholesterol depletion is not a result of the remaining cholesterol being located at the interior of the membrane and being inaccessible to the plasma lipoproteins. The fact that no more than 40% of the cholesterol is removed, over the wide range of conditions examined, suggests that cholesterol may exist in two states in the membrane, one freely exchangeable and the other firmly bound. Future plans call for utilizing fluorescent polyene antibiotics, which are known to complex with cholesterol, to explore this.

The practical significance of fundamental work on the role of lipids in cell membranes was originally considered to be in the context of the various lipid storage diseases. In the light of the recent reports by the Israeli workers, Inbar and Shinitsky, that the cholesterol content of lymphocyte membranes may be a factor in leukemia, the present work also may have significance for cancer research.

Keyword Descriptors:

membranes, cholesterol, erythrocytes, lipoproteins, blood plasma, cholesterol esters

ublications:

Gottlieb, M. H. and Eanes, E. D.: On phase transitions in erythrocyte membranes and extracted membrane lipids. *Biochim. Biophys. Acta* *373*: 519-522, 1974.

Reported in Press - 1974

Gottlieb, M. H.: Binding of alkaline and alkaline earth cations in lecithin mesophases. *J. Colloid Interface Sci.* *48*: 394-399, 1974.

Project No. Z01 AM 27005-21 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Investigation of the macromolecular organization of living matter.

Previous Serial Number: NIAMDD-LPB-6

Principal Investigator: L. W. Labaw

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives:

To gain information about the macromolecules that are essential constituents of living matter and to see how they are arranged in the structures they form. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near its limit of resolution.

Methods Employed:

Electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. Physicochemical characterization of macromolecular components isolated from such material using electron microscope, X-ray diffraction, and similar established techniques.

Major Findings:

An orthorhombic crystal of Fab' fragments of a mouse myeloma immunoglobulin protein has been examined in the electron microscope. The crystals were grown by Dr. David R. Davies' group and are currently being studied by them using X-ray diffraction. Their preliminary work showed these Fab' crystals to belong to the $P2_12_12_1$ space group with unit cell dimensions of a = 54.2, b = 74.4, and c = 131.5 Å. The data from the EM study agreed with these unit cell dimensions within 7% and gave projections showing the approximate locations of the molecules which were useful in making the low resolution X-ray analysis.

In a collaborative study with Dr. Leo Levenbook, sections through the larval fat body of the blowfly have been examined in the EM. Many of the non-fat structures therein show crystalline arrays. These were extensively studied to determine the characteristics of the particles forming the arrays. The particle was found from the determined crystal packing to be an approximate sphere about 132 Å in diameter, having a core about half this diameter which is weakly self staining. Unlike the iron core in ferritin particles, this core is not apparent in individual particles but is seen only when several particles are aligned in the electron beam direction. These particles are thought to be the protein calliphorin which contains calcium. This interpretation fits the EM data, and attempts are now being made to crystallize purified calliphorin to see if it looks the same in the EM.

Significance to Biomedical Research and the Program of the Institute:

The use of the electron microscope to determine the crystal structure of protein crystals is particularly important when applied to crystals which cannot be grown large enough for a more complete structure study by X-rays or which are too sensitive to X-ray degradation.

With increased emphasis on fine structure in electron microscopy, it is pertinent to examine the mechanism of image formation to define the conditions under which one can be assured that observed periodicities on images in the electron microscope have a direct spatial relationship with the object being examined when one is operating near the resolution limit of the instrument.

Proposed Course of Project:

To investigate other protein crystals in the electron microscope using replica, negative staining and direct transmission techniques to determine their structures.

Keyword Descriptors:

protein crystals, electron microscopy, myeloma proteins, calliphorin

Publications:

Labaw, L. W., Padlan, E. A., Segal, D. M. and Davies, D. R.: An EM study of phosphorylcholine-binding Fab' immunoglobulin fragment crystals. J. Ultrastruct. Res., in press.

Project No. Z01 AM 27006-18 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical Changes during Development of the Blowfly

Previous Serial Number: NIAMDD-LPB-7

Principal Investigator: L. Levenbook

Other Investigators: A. Bauer and A. Protzel

Cooperating Units: Dr. Andreas Chrambach
Dr. Marc S. Lewis EI-LVR-

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives:

1. An elucidation of the structure and function of the insect protein calliphorin.

2. To help set up an insect biochemical laboratory in Cairo, Egypt and elucidate the biochemistry of ticks.

3. An understanding of the interrelationship(s) of the ribonucleic acids of a polymorphic developing system as illustrated in blowfly metamorphosis.

4. To determine the molecular weights of some tick proteins.

5. To determine the crystalline structure of calliphorin *in vivo* and *in vitro*.

6. Publication of the mass of data accumulated on *Phormia regina* fat body aldolases.

Methods Employed:

Routine physical methods for the isolation of ribosomes, RNA, precursor pools, and calliphorin, molecular weights and crystal structure.

Routine chemical procedures for the study of ribosomes, RNA, and purified proteins.

Major Findings:

1. The best method for the purification of calliphorin has been found to be the use of the Ortec preparative electrophoresis and Chrambach system 3227 (25°C, pH 6.5) as the last step. Eighty percent of the protein put on the gel can be recovered. Over 50 milligrams of pure calliphorin are on hand for study.

2. It has been found that tick blood contains tyrosinase, a fact that had been previously denied.

3. The pool of ribosomal RNA remains essentially constant (Ave. 200 μg) during metamorphosis. There is a low value (170 μg) during the early pupal stage and a high value (230 μg) during the late pupal stage. The pool of ribosomal RNA turns over with a half life of 8.5 days. This value corresponds to the rate of synthesis of 15 μg of rRNA/day/insect. If the specific radioactivity of the precursor pool is considered, a rate of synthesis of 36 μg of rRNA/day/insect may be estimated. The pools of 18S and 28S ribosomal RNA turn over with statistically undistinguishable half lives, thus suggesting similar degradation rates for both ribosomal subunits. Injection of radioisotopes into blowfly pupae allowed direct measurement of the rates of ribosomal RNA synthesis during metamorphosis. The rates of synthesis obtained thus are from 2.0 to 4.8 times higher than the values obtained from the turnover studies of ribosomal RNA. These higher values may indicate that the UTP pool which provides the immediate precursors for RNA synthesis behaves as a small compartment in rapid equilibrium with the externally supplied nucleotide. The rates of synthesis of ribosomal RNA obtained both through turnover studies and by direct measurements indicate that at least 50%, and possibly most, of the ribosomes in the adult blowfly have been synthesized de novo during metamorphosis. Thus, there is little or no carry-over of ribosomes from the larval to the adult stage.

4. The tick proteins were obtained by column chromatography in Egypt. Fraction A and Fraction B proteins were prepared for ultracentrifugation and taken over to Marc Lewis to run on the ultracentrifuge (analytical). Each fraction contained two proteins. The molecular weights of the proteins in fraction A were 0.5×10^6 daltons and 3.5×10^6 daltons. Those of Fraction B were 0.75×10^6 and 8.6×10^6 daltons.

5. Sections of fat body from different stages of the calliphora maggot and pupa were fixed and taken to Charles Hanna for sectioning. Louis Labaw elucidated the structure of the crystals found in the sections.

Keyword Descriptors:

Calliphorin
Ribosomal RNA
Blowfly RNA
Blowfly
Ribosomes
Tick Protein

Significance to NIAMDD Research:

Insect metamorphosis is a programmed process under hormonal control which entails the decay and destruction of the larval cells concomitantly with the differentiation of the cells and structures that will give rise to the adult insect. The greater part of this process has taken place within the span of a week. This telescoping of the aging and development process in the insect might be expected to bring forth features otherwise unobservable in the corresponding processes of man and mammals, which take place in a much more expanded time scale. In particular, the study of the fate of proteins, ribosomes and ribosomal RNA refers to the fate of essential components of the protein synthesizing machinery and its products during aging and development.

Proposed Course of Project:

Continuation of studies on developmental biochemistry of ribosomal RNA and poly-A containing RNA in the blowfly, and the structure and function of calliphorin. Study of factors involved in destruction of tissues by phagocytic blowfly hemocytes.

Publications:

Bauer, A. C., Shigematsu, H., and Levenbook, L.: Comparison of larval and adult muscle aldolases of the blowfly Phormia regina. Insect Biochem. 4: 123-133, 1974.

Project No. Z01 AM 27007-13 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Molecular Mechanism of Human Red Cell Sickling and Unsickling *in vitro* and *in vivo*.

Previous Serial No.: NIAMDD-LPB-8

Principal Investigator: Makio Murayama

Other Investigators: Dr. Alan S. Levine

Cooperating Units: Dr. Oku Ampofo, Tetteh Quarsh Memorial Hospital and Ministry of Health, Mampong-Akwapim, Ghana, West Africa

Dr. Sidney Shifrin and Mrs. Catherine L. Parrott, Laboratory of Cell Biology, National Cancer Institute, Z01 CBD 05543-07

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

1. To find a molecular mechanism of "unsickling" of erythrocytes from sickle cell anemia patients.

2. To evaluate thermodynamic constants: the enthalpy change in the thermal (endothermic) aggregation of sickle cell hemoglobin (Hb S); the entropy change in the thermal aggregation of Hb S; and the volume of activation in the endothermic aggregation of Hb S.

3. To investigate further the effects of the constituents of the Hofmeister Series on the blockage and reversal of sickling using S hemolysate and S erythrocytes.

4. To study the effects of inhibitors of microtubule formation because:

   a. Hb S forms microtubule upon sickling.
   b. There is evidence for hydrophobic interactions being responsible

for Hb S microtubule formation.
  c. Microtubules in general are formed by hydrophobic interactions.

5. To investigate a co-factor of S Hb aggregation.

Methods Employed:

Differential interference (Nomarski) optics microscopy using a chamber which is adequate for high resolution light microscopy for time lapse cinephotomicrography of sickling and unsickling process; in addition observations are recorded on magnetic video tape for future transcribing only the desired "strips" onto 16 mm film. Other methods are essentially the same as reported previously.

Major Findings:

Sickle cell hemoglobin when deoxygenated has great propensity to aggregate; this aggregation can be inhibited by inorganic ions according to the classical Hofmeister series (Cl^- NO_3^- Br^- I^- SCN^-). Essentially the same results were obtained by the thermodynamic solubility tests in a phosphate buffer. The solubility test was used to investigate the influence of monohydric and polyhydric alcohols on aggregation of sickle cell hemoglobin. It was found that the effectiveness of a series of polyhydric alcohols to promote aggregation increases as the number of hydroxymethyl groups about the asymmetric carbon atoms also influences the degree of aggregation.

Keyword Descriptors:

Sickle Cell Disease
Sickle Cell Anemia
Deoxy-S-hemoglobin Aggregation
Molecular Mechanism of Human Red Cell Sickling
Hydrophobic Interactions
Desickling
Solubility of Hemoglobins
Hoffmeister Series on Desickling
Oral Urea Therapy
Mono and Polyhydric Alcohols on Aggregation of S Hemoglobin

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, hemolytic anemia -- an inborn error of metabolism, hence significant to the NIAMDD mission. My theory of hydrophobic interaction as the root cause of human red cell sickling has now been confirmed by nearly a dozen different laboratories. My theory has been tested at the bedside with success in Michigan as well as in Africa (oral urea therapy and oral urea prophylaxis), Sao Paulo, Brazil, and Havana, Cuba. When urea therapy has been administered with no intellectual understanding of the theory behind urea, as in the instance of NIH-NHLI cooperative study, this could lead to failure.

Proposed Course of Project:

Inorganic ions of Hofmeister series will be studied to find out whether or not any combination of two or more salts would yield an additive effect. If this would be true, then sodium iodide and sodium thiocyanate could be used with impunity (sodium thiocyanate would decrease the activity of the thyroid gland whereas sodium iodide will accerate same).

Honors and Awards:

Medical Advisor to Sickle Cell Anemia Research and Education, Inc.,
2201 Steiner at Sacramento, San Francisco, California 94115.

Scientific Advisor to International Sickle Cell Anemia Research Institute, Mampong-Akwapim, Ghana, West Africa (attached to Ministry of Health of Ghana).

Publications:

Levine, A. S., Hasegawa, F., and Murayama, M.: The influence of solutes and solvent structure on aggregation of deoxy-sickle cell hemoglobin. J. Mol. Med. In press.

Shifrin, S., Levine, A. S., and Parrott, C. L.: The influence of monohydric and polyhydric alcohols on aggregation of sickle cell hemoglobin. J. Mol. Med. In press.

REPORTED IN PRESS - 1974

Murayama, M.: Molecular aspects of tactoid formation in man and animals. Ann. N.Y. Acad. Sci. 251: 623-637, 1974.

Project No. Z01 AM 27008-16 LPB
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of metabolic activity in microorganisms

Previous Serial Number: NIAMDD-LPB-5

Principal Investigator: Ellis S. Kempner

Other Investigators: Jay H. Miller and Talaat E. Shehata

Cooperating Units: None

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Microscopic examination of cells by time-lapse video micrography.

Major Findings:

Evidence for the sub-cellular localization of enzymes in Euglena and many other organisms has led to the idea of biochemical organization of entire pathways; reports of the non-uniform utilization of exogenous and endogenous substrates has further supported these ideas. Consideration of the simplest forms of physical binding of enzymes and substrates leads to predictable and testable properties of organized metabolism. Several experimental systems appear to fit the predicted models surprisingly well. Attempts will be made to identify organized biochemical pathways in Euglena and test the predicted characteristics in these systems.

Previous studies of Euglena gracilis had shown a strong homeostatic control of the amino acid pool, independent of the external environment (culture medium composition). The abundance of amino acid "family heads" such as glutamic acid, aspartic acid and glycine was observed under conditions which suggested that amino acid biosynthesis might occur by bound intermediates -- a very effective mechanism for hemeostatic regulation. The ultimate fate of these compounds is of course the cellular protein, and its composition should reflect still further regulation. Unfortunately the hydrolysis of peptide bonds requires conditions which can destroy various amino acid residues. A variety of hydrolytic procedures was therefore used in order to obtain quantitative recoveries of each amino acid. Five amino acids -- glutamic acid, leucine, alanine, aspartic acid and glycine -- account for half the residues of gross Euglena protein and 80% of the amino acid pool. Considerable attention was given to the determination of protein tryptophan which was found to be small, 0.5% of the molar content.

Vitamin B12, needed by man to prevent anemia, is required by Euglena gracilis in such small amounts that the protozoan growth is used as a bioassay for the vitamin. Although the biochemical activity has been studied in many organisms, the role of the vitamin B12 in Euglena metabolism is unknown. Cultures deprived of the vitamin show a doubling time of 25-26 hours instead of the normal 12-13. After long periods of starvation the growth slows further and eventually stops. The cell volume distribution changes in shape and value, and the mean volume increases gradually from 3000 μ^3 to about 8000 μ^3 after 240 hours of starvation. There is a simultaneous increase in cellular protein (2 1/2-fold), RNA (2-fold) and DNA (not quite double). These preliminary results indicate that vitamin B12 starvation inhibits cell division of Euglena, probably at a late stage of the division process.

Keyword Descriptors:

Growth of Microorganisms
Metabolic Control Mechanisms
Protein Composition

Significance to NIAMDD Research:

This work is aiding the development of a system for studying differentiated cellular structure in Euglena gracilis with the technical ease usual with bacteria. Using this system, new insight into the structure and function of cells is being gained.

Proposed Course of Project:

To study the metabolic relationships of synthetic processes in Euglena to the highly organized cellular structure which has already been elucidated.

Publications:

REPORTED IN PRESS - 1974

Kempner, E. S. and Miller, J. H.: The molecular biology of Euglena gracilis. IX. Amino acid pool composition. J. Protozool. 21: 363-367, 1974.

ANNUAL REPORT OF THE
LABORATORY OF BIOPHYSICAL CHEMISTRY, NIAMDD

The main objective of this laboratory has been, as in previous years, the study of the correlation of structure of protein molecules to their functions. The proteins studied were the oxygen-carrying proteins, those participating in muscular contraction, and blood clot formation. To varying extent, the primary, secondary and tertiary structure of these proteins came into the field of our inquiries as the following summary of the work reported illustrates.

MUSCULAR CONTRACTION: Experiments carried out in the Section on Bioenergetics are aimed at correlating enzymatic activity and physical properties of purified contractile and regulatory proteins, of cell suspensions, and of glycerinated muscle fibers. A sensitive fluorimetric technique has been developed for rapid measurement of ATPase of small bundles of glycerinated fibers while being monitored for length and tension changes.

Binding studies suggest a dual role for the regulatory protein of skeletal muscle, troponin-I. First, it induces tropomyosin to bind to F-actin, causing tropomyosin to inhibit the actomyosin ATPase. Second, it can itself enhance the inhibition of the ATPase. It appears that troponin-C, upon binding to troponin-I, can block the second of these actions, but not the first. (Kominz, Eaton, Eisenberg).

Muscle is an energy transducer and converts chemical energy stored in ATP into mechanical work. Myosin and actin organized into separate filaments represent the actual machine. Current theories depict the globular head of the myosin molecules as the site of force generation. As we have pointed out earlier, the globular portion of myosin shows great similarity to actin; thus, in our opinion, actin (F-actin) should be examined as a possible force-generating structure. And since actin under certain conditions cleaves ATP stepwise, it offers a simplified system for the study of how ATP is converted to mechanical work. Utilizing the known amino acid composition of actin, its resemblance to other nucleotide-binding proteins and also our experimental work which implicates an Arg(28) and a Lys(113) residue in the binding of the γ-phosphate of ATP, we constructed a three-dimensional structure of the ATP binding domain of actin. With that model, we can suggest that an essential step in energy transduction is a change of ATP into a low energy state with the concomitant energization of the protein structure. This high energy state of the protein is visualized as a strain in the β-pleated sheet or in a distorted helical region. (Laki, Ladik).

In order to examine whether ATP binding to actin's Arg and Lys residues leads to a low energy ATP, CNDO/2 (complete neglect of differential overlaps) calculations are being carried out.

HEMOGLOBIN: The kinetics of oxidation of hemoglobin by hydroxylamine and by oxygen are being studied. It was found that in a closed vessel oxyhemoglobin in solution is converted mainly to deoxyhemoglobin, rather than methemoglobin, indicating that there is an oxidation of something in the hemoglobin at a much faster rate than oxidation of the heme.

Binding data on hemoglobin allows an analysis of the cooperative effect in terms of the sites at which the energy of interaction is stored. Deoxyhemoglobin is a relaxed molecule (from the standpoint of the interaction energy) while oxyhemoglobin is raised to a higher potential energy level by an energetic increment equal to the interaction energy. The oxygenation data at different concentrations of protons and anions are being analyzed in conjunction with differential titration data for hemoglobin in order to quantitate the increment of interaction energy related to the pH dependent, or Bohr sites. (Saroff, Simpson).

The understanding of the process by which sickle-cell hemoglobin (HbS) aggregates is expected to lead to a knowledge of how to inhibit the aggregation and thereby alleviate the symptoms of sickle-cell disease. The equilibrium thermodynamic model for aggregation ("gelation") of HbS hemoglobin in the presence of additives has been extended in several important respects. Previous assumptions regarding the relative affinity of an additive for aggregated and non-aggregated HbS were eliminated. The resulting general treatment is capable of satisfactorily accounting for currently available data on the inhibition of HbS gelation by a wide variety of additives in a unified manner. The equilibrium model has been used to deduce information about the relations between oxygenation and aggregation from literature data on the oxygenation of sickle erythrocytes under controlled conditions. These extensions of the thermodynamic model for HbS gelation constitute a substantial step forward in our understanding of HbS aggregation under physiological conditions. The new instrument reported earlier for automatic measurement of the minimum gelling concentration of HbS solutions has demonstrated its superiority in speed, reproducibility, and ease of measurement over the conventional method of performing this measurement. (Minton).

FIBRINOGEN-FIBRIN TRANSITION: The investigation involves the protein-protein interaction concerned with blood coagulation. This is oriented particularly to the molecular biological mechanisms of the thrombin conversion of fibrinogen to fibrin plus the ensuing enzymatic cross-linking of fibrin monomers to form the actual physiological material constituting a "blood clot".

We are studying lamprey blood coagulation in the hopes that any anomalies encountered may in actuality point up heretofore unexplained coagulation anomalies reported in man.

Close attention has been focused on the further elucidation of the cross-linking mechanisms of lamprey fibrinogen and fibrin. It appears that the lamprey α-chains serve no important function in cross-linking; i.e., they're not involved in lamprey blood clotting. This is in contrast to human coagulation where complete cross-linking of α-chains is mandatory to obtain the stable clot. Release of A-peptide from lamprey α-chain is not necessary for clotting; the release of B-peptide by thrombin is sufficient, not only to initiate clotting, but subsequent cross-linking as well.

The establishment of the molecular parameters and unit structure of lamprey fibrinogen is important. Equilibrium centrifugation gives a molecular weight which is more consistent with that found with fibrinogens of the higher species, 330 to 350 thousand daltons. (Gladner, Murtaugh, Lewis).

It is now becoming realized worldwide that two enzymes, thrombin and transglutaminase (activated Factor XIII), must act on fibrinogen to produce a normal clot. If plasma transglutaminase is inoperative, a bleeding tendency arises which in children leads to fatal intracranial bleeding. In addition to functioning in hemostasis, Factor XIII is also involved in wound healing. We have reported animal experiments indicating that transglutaminase is also involved in tumor growth and metastasis. Our recent findings suggest that transglutaminase also plays a role in the metastasis of human tumors. It was found that the frequency of metastasis into the host organs correlates with the transglutaminase content of the organs. The mechanism responsible for the correlation of transglutaminase and tumor metastasis is under investigation. (Laki, Yancey, Chung).

Serial No. Z01 AM 31000-15 LBC
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Proteins, enzymes and peptides involved in blood coagulation. Proteases of pituitary glands and their possible relationship to pituitary hormones.

Previous Serial No.: NIAMDD-LBC 4

Principal Investigator: Jules A. Gladner

Other Investigators: Mrs. Patricia A. Murtaugh

Cooperating Units: Dr. John Halver, Chief, Fish Nutrition Lab., Fish and Wildlife Service, Dept. of Interior, Cook, Washington; Dr. Marc S. Lewis, NEI, NIH; Dr. Peter Condliffe, NIAMDD-LNE; Dr. L. Kohn, NIAMDD-LBP; Dr. S. I. Chung, NIDR-LB.

Man Years:
Total: 4.5
Professional: 4.5
Others: 1

Project Description:

Objectives: The major investigation involves the protein-protein interaction concerned with blood coagulation. This is oriented particularly to the molecular biological mechanisms of the thrombin conversion of fibrinogen to fibrin plus the ensuing enzymatic cross-linking of fibrin monomers to form the actual physiological material constituting a "blood clot". In addition, we have begun investigating the possible enzymatic reversal of this cross-linking reaction.

Another possible type mechanistic study has begun involving an investigation of the proteases of the pituitary gland. Past reports in the literature have reported large molecular weight pituitary hormones. Our object is to ascertain if there exist pro-pituitary hormones and if pituitary proteases, of the limited protease variety, play a role in converting pituitary pro-hormones to an active or more active type.

Methods: Methods employed consisted of protein-enzyme fractionation, purification, characterization and modification. Where needed, methods of physical biochemistry and analytical chemistry were utilized, such as, but not limited to, SDS gel electrophoresis and automatic amino acid analysis.

Physiological techniques were utilized to examine the biological activity of various peptides. As applicable, new techniques were developed.

Major Findings: Cross-linking of Fibrinogens and Fibrins of Lower Species. Close attention has been focused on the further elucidation of the cross-linking mechanisms of lamprey fibrinogen and fibrin, as well as the accurate determination of the molecular weights of these proteins. The following information has emerged:

(1) Lamprey fibrinogen and fibrin appear to differ in their molecular properties from any of those proteins studied in higher species. Although these proteins still consist of six chains possessing the formulation $\alpha_2\beta_2\gamma_2$, their reaction towards cross-linking via Factor XIII, although resembling those of higher species, differs in some major aspects. In order to investigate the above, it was necessary to render purified lamprey fibrinogen, 93-95% clottable or better, free of its intrinsic Factor XIII. This was accomplished with great difficulty; however, the results obtained with this material gave a clearer indication of its subsequent role in cross-linking reactions. It appears that the lamprey α-chains serve no important function in cross-linking; i.e., they are not involved in lamprey blood clotting. This is in direct contrast to human coagulation where complete cross-linking of α-chains is mandatory to obtain the most stable clot. Other sharp contrasts exist. Release of A-peptide from lamprey α-chain is not necessary for clotting; the release of B-peptide by thrombin is sufficient, not only to initiate clotting, but subsequent cross-linking as well. A stable clot results when only γ-chains of lamprey species are cross-linked regardless of the amount of α-chains cross-linked. Lysis times of cross-linked lamprey blood clots are a function of the degree of γ-chain cross-linking; the α-chains appear to play little or no role. In fact, α-chains cross-link at an extremely slow rate and complete α-chain cross-linking via intrinsic lamprey Factor XIII has not been possible. Only the addition of human Factor XIII will completely cross the α-chains; yet it must be stressed, as stated above, that the lamprey α-chains seemingly play no role in clot stability or lysis times. Thus, in lamprey, the first of the vertebrates, the β- and γ-chains of the fibrinogen appear to be the important chains in the clotting mechanism. The α-chain, although in existence, seems to assume its major role further along the evolutionary ladder. (Murtaugh, Gladner, Halver).

(2) Initial investigations aimed at isolation of lamprey Factor XIII have been instituted. A startling observation is that the level of lamprey plasma Factor XIII activity utilizing the dansyl cadaverine incorporation assay system is about one-thousandth of that found in human plasma. Yet, as previously discussed, it is more than sufficient to form stable cross-linked fibrin clots. These studies are proceeding slowly since lamprey plasma is difficult to obtain in large quantities, and then only one yearly. (Gladner, Chung).

(3) The establishment of the molecular parameters and unit structure of lamprey fibrinogen is also an important facet of our overall investigations. When polyacrylamide gels are utilized to ascertain molecular weights,

we, as other investigators, are in agreement with a molecular weight of about 460 thousand daltons. However, utilization of methodology based on firm thermodynamic concepts, i.e., equilibrium centrifugation, give a molecular weight which is more consistent with that found with fibrinogens of the higher species, 330 to 350 thousand daltons. The crux of the entire matter lies in the establishment of the true molecular weight of the α-chain. This is now being studied very closely. (Gladner, Lewis); (Gladner, Chung).

In general, we are studying lamprey blood coagulation in the hopes that any anomalies encountered may in actuality point up heretofore unexplained coagulation anomalies reported in man. Lamprey plasma itself requires exceedingly careful manipulation. Our experiments have shown, in sharp contrast to reports published by other investigators, that (a) the fibrinogen is easily degraded during isolation, particularly the α-chain and (b) during isolation and purification, the purer the fibrinogen obtained, the more easily it is spontaneously converted to fibrin by traces of thrombin impurities. One can only postulate that this effect is due to the removal of natural protein inhibitors of the various intrinsic and extrinsic coagulation factors which ultimately result in the formation of thrombin. Again this may prove of value for better understanding of coagulation mechanisms in higher species, man in particular.

Enzymatic reversal of fibrin cross-linking. As previously reported, an enzyme "procollagen peptidase", responsible for conversion of procollagen to collagen, appears to be capable of rupturing the γ-glutamyl-ϵ-amino lysyl bonds of the cross-linked α-chains of urea insoluble fibrin; i.e., a physiological type clot. Closer examination indicates the occurrence of more than one enzyme in the preparation, one which may be proteolytic and responsive to DFP. The procollagen peptidase is not inhibited by DFP and experiments are in progress to further delineate its possible utilization as a "decross-linking" agent on fibrin clots. (Gladner,Kohn).

Proteases of the pituitary. Further investigation employing more susceptible substrates (azocasein) has been instituted to investigate the unusual activity of pituitary proteolytic enzymes. Their nature and function are unknown. They display an unusual activity in that DFP does not appear to cause any drop in activity. Thus far, the actual number still remains to be discerned, as well as the possible role of activators, metal ions, GSH, etc. (Gladner, Condliffe).

Keyword Descriptors: Blood coagulation, cross-linking, proteolytic enzymes, proteins, peptides, pituitary.

Significance to biomedical research and the program of the Institute: By the utilization of blood systems of lower species, one might observe reactions which are masked or even only slightly residual in man, but a malfunction of same could lead to undefined reasons for plasma component(s) maladies. Any information concerning thrombin action is most vital to eventual regulation of the action of this enzyme.

Proposed course of project: We anticipate proceeding along the lines outlined in the above report with emphasis placed on major results as they occur.

Honors and Awards: None.

Publications:

1. Houck, J. C. and Gladner, J. A.: Proposed mechanisms for the ameliorative effects of corticosteroids in shock. In *Shock: Biochemical, Pharmacological, and Clinical Aspects*. Plenum Press, 1970, pp. 125-132.

2. Murtaugh, P. A., Halver, J. E., Lewis, M. S. and Gladner, J. A.: Cross-linking reactions of lamprey fibrinogen and fibrin. *Biochim. Biophys. Acta* 359: 415-420, 1974.

3. Murtaugh, P. A. and Gladner, J. A.: A modified procedure for the rapid isolation of fibrino-peptides. *Arch. Biochem. Biophys*. 2: 784-786, 1974.

4. Muszbek, L., Gladner, J. A. and Laki, K.: The fragmenetation of actin by thrombin. Isolation and characterization of the split products. *Arch. Biochem. Biophys*. 167: 99-103, 1975.

Serial No. Z01 AM 31001-3 LBC
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mechanisms of Muscular and Cellular Contraction

Previous Serial Number: LBC-1

Principal Investigator: David R. Kominz

Other Investigators: Barbra Eaton
Henry Wolff

Outside Collaborators: Evan Eisenberg, LB, NHLI
Ned Feder, LEP, NIAMDD

Man Years:

Total: 3.5
Professional: 2.0
Others: 1.5

Project Description:

Objectives:

To advance understanding of muscular contractility.

Methods:

Experiments aim at correlating enzymatic activity and physical properties of purified contractile and regulatory proteins, of cell suspensions, and of glycerinated muscle fibers. Studies of ATPase activity in the pH-stat have been continued. A sensitive fluorimetric technique has been developed for rapid measurement of ATPase of small bundles of glycerinated fibers being monitored for length and tension changes. Methods for production of large quantities of standard rods for accurate length and volume measurements have been developed.

Major findings:

1. With a coupled system of pyruvate kinase and lactate dehydrogenase, it was possible to measure the ATPase activity of small bundles of glycerinated muscle fibers by following the disappearance of NADH fluorescence for 2-4 minutes. This makes it possible to correlate ATPase with tension or work

performed by oscillating muscle preparations over a large range of frequencies, ATP concentrations and temperatures (Kominz).

2. Binding studies suggest a dual role for the regulatory protein of skeletal muscle, troponin-I. First, it induces tropomyosin to bind to F-actin, causing tropomyosin to inhibit the actomyosin ATPase. Second, it can itself enhance the inhibition of the ATPase. It appears that troponin-C, upon binding to troponin-I, can block the second of these actions, but not the first (Eaton, Kominz and Eisenberg).

3. Improved procedures allowed the preparation of large quantities of microscopic rods of uniform length from commercial nylon thread of 12-16μ diameter. The failure of long rods (longer than orifice diameter but shorter than orifice length) to yield volumes within expected limits was found to be a measure of instrumental inadequacy rather than weakness of the theory (Kominz, Wolff and Feder).

Projects Proposed:

1. Using binding and other studies, we shall extend our examination of the interaction between muscle proteins from actin-heavy meromyosin to actin-myosin and myofibrils which contain actin and myosin in a structured hexagonal array.

2. Possible correlations will be examined between ATPase measurements on glycerinated fibers and measurements of tension and work obtained at the same time.

3. Suspensions of intact cells will be prepared from various sources, and their contractile response to pharmacological and other agents will be examined.

Significance:

There are still areas of uncertainty concerning the interaction of the contractile and regulatory proteins of muscle. Although proteolytic enzymes simplify some studies of these interactions, the modified muscle proteins have certain properties not found in the native state. It is therefore useful to extend our studies to preparations which have not been degraded by proteolytic enzymes. This will allow our definition of the normal state to be more exact, and hopefully will facilitate definition of deviations from the normal which are found in disease.

Keyword Descriptors:

Muscle contraction, tropomyosin, actin, troponin, actomyosin ATPase

Publications:

Eaton, B. L., Kominz, D. R., Eisenberg, E. : Correlation between the Acto-heavy meromyosin ATPase and the binding of tropomyosin to F-actin; in press, Biochemistry

Serial No. Z01 AM 31002-4 LBC
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Intermolecular Interactions in Physical Biochemistry

Previous Serial Number: LBC-5

Principal Investigator: Allen P. Minton

Man Years:
Total: 1.0
Professional: 1.0

Project Description:

Objectives:

1. To increase our knowledge about and understanding of the process by which sickle-cell hemoglobin aggregates; and to use this knowledge and understanding to develop chemical means for inhibiting the aggregation of sickle-cell hemoglobin *in vivo*, thereby alleviating symptoms of sickle-cell disease.

2. To ascertain the structural basis of cooperative ligand binding in hemoglobin.

Methods:

Theoretical studies employ methods of classical and statistical thermodynamics together with computer techniques for fitting theoretical model functions to experimental data. Experimental studies utilize an automated viscosity-based assay for sickle-cell hemoglobin aggregation which was described in last year's report, and a novel optical method, developed by L. Liesow and D. Nelson, of measuring the fractional oxygen saturation of dilute solutions of hemoglobin and suspensions of erythrocytes as a function of concentration of dissolved oxygen.

Major Findings and Proposed Course of Studies:

The thermodynamic model for aggregation ("gelation") of sickle-cell hemoglobin (HbS) in the presence of additives, which was described in last year's report has been extended in three important respects.

a. Previous assumptions regarding the relative affinity of an additive for aggregated and non-aggregated HbS were eliminated and the resulting general treatment is capable of satisfactorily accounting for currently available data on the inhibition of HbS gelation by a wide variety of additives in a unified manner.

b. The effect of change in protein conformation accompanying the binding of heme-ligands has been taken into account by means of a detailed statistical treatment of near-neighbor interactions in the HbS aggregate. The resulting equilibrium model has been used to deduce information about the relations between oxygenation and aggregation from literature data on the oxygenation of sickle erythrocytes under controlled conditions.

c. The possibility of non-equilibrium contributions to the observed data on oxygenation of sickle erythrocytes has been explored and a kinetic extension of the equilibrium model outlined above has been proposed. The extended model has been found to account in a natural way for those features of the oxygenation data which were not readily interpreted in the context of an equilibrium model.

Last year we reported the construction of a prototype instrument for automated measurement of the minimum gelling concentration (MGC) of HbS solutions. In collaboration with N. Rumen of the Albert Einstein Medical Institute, Philadelphia, this instrument has been used to measure the effect of various amino acids upon the gelation of HbS. In collaboration with I. Tice of the BEIB Instrumentation Facility, a "production model" of this instrument, incorporating several improvements in design and providing a six-fold increase in capacity, has recently been completed and checked out.

In collaboration with L. Kiesow and D. Nelson of the Naval Medical Research Institute, experiments to measure the dependence of oxygenation in sickle erythrocytes upon the intracellular concentration of HbS and 2,3-diphosphoglyserate have been initiated.

The energetics of oxygen binding to cobalt-substituted hemoglobin have been analysed and compared with those of normal (iron) hemoglobin. It has been shown that the relationship between structural changes at the heme accompanying oxygen binding and the concomitant changes in the free energy of interaction are highly non-linear, in contrast to published speculations.

During the coming year our studies will focus on the following areas:

1. Continuation of the collaborative studies referred to above, to acquire additional data on the effects of oxygen and various additives upon the aggregation of HbS.

2. Continuation of studies of cooperative ligand binding in hemoglobin.

3. A combined experimental and theoretical study of the self-association of retinol and retinalhyde in non-polar solvents is being planned in collaboration with M. Lewis of the Laboratory of Vision Research, NEI.

Significance to Biomedical Research and the Programs of the Institute:

The extensions of the thermodynamic model for HbS gelation constitute a substantial step forward in our understanding of HbS aggregation under physiological conditions. Furthermore, the general relations derived in these studies are applicable to a broad range of macromolecular self-assembly processes in biology, such as the assembly of neurotubules from tubulin.

Measurement of the MGC of HbS solutions remains one of the most important and reliable ways of assaying the effects of additives upon the aggregation of HbS. The new instrument for automatic measurement of the MgC has demonstrated its superiority in speed, reproducibility, and ease of measurement over the conventional method of performing this measurement.

Keyword Descriptors:

Hemoglobin oxygenation, sickle-cell hemoglobin gelation.

Publications:

1. Minton, Allen P., and Saroff, H. A.: A general formulation of the free energy of interaction in cooperative ligand binding: Application to hemoglobin. Biophys. Chem. 2, 296 (1974)

2. Minton, Allen P.: Chemical inhibition of sickle-cell hemoglobin gelation: A thermodynamic analysis. In press, J. Mol. Biol.

Serial No. Z01 AM 31003 1 LBC
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The action of thrombin on fibrinogen and the physiological mechanism of action of the peptides released during blood clotting.

Previous Serial No.: NIAMDD LBC-3

Principal Investigator: A. J. Osbahr

Man Years:

Total: 1.0
Professional: 1.0

Project Description:

Objectives:

An improved understanding of the structural basis for the enzymatic action of thrombin on fibrinogen, and for the physiological action of the peptides released from fibrinogen during blood clotting, in order to gain insights into the possible role of these peptides in hemostasis, vasoconstriction and pulmonary hypertension.

Methods:

Homogeneity and purity of the protein and peptide systems were evaluated with ultracentrifugation and regular and SDS gel electrophoresis while peptide structure and composition were analyzed by high voltage electrophoresis,liquid column chromatography and spectrophotometric analysis.

Proteolytic enzymes were employed for "active site" determinations. Physiological monitoring was performed on rats by means of intracarotid cannulation or microscopic visualization of bleeding arterioles of scarified rat tail.

Major Findings and Proposed Course of Study:

The number of bonds hydrolyzed by the enzymatic action of canine thrombin on canine fibrinogen was studied as a function of time. In one hour approximately 1.5 bonds are enzymatically hydrolyzed by thrombin; 2.5 more bonds are slowly hydrolyzed over the next 6-7 hours. This slow hydrolysis of peptide bonds from fibrinogen may be explained by the structure relationship of the fibrinopeptides that regulates the order and speed with which they are proteolytically removed from canine fibrinogen.

The canine fibrinopeptides were enzymatically hydrolyzed by pepsin to produce peptide fragments which will be utilized to investigate the relationship between biological activity and structure in a study of the physiological role of these peptides in hemostasis, vasoconstriction and pulmonary hypertension. The peptic fragments were separated on a Dowex 50 (X-2) column and purified by chromatography and electrophoresis.

The modification of bovine fibrinogen via trimethyl oxonium fluoroborate and dimethyl sulfate resulted in the polymerization of fibrinogen over a 1-2 hour period. The modified fibrinogen was solubilized in 6M urea and 2N acetic acid and repolymerized by dialyzing out the 6M urea.

Significance of Biomedical Research and the Program of the Institute:

Our studies indicate a relationship between the peptides from the blood clotting protein fibrinogen and physiological processes in blood circulation. It is hoped that investigation of these physiologically active peptides may result in a deeper understanding of the mechanisms associated with hemostasis, vasoconstriction and blood coagulation; and could provide clues to the etiology of certain diseases associated with high blood pressure such as respiratory distress syndrome. The presence of fibrinopeptide may represent a possible agent for such vasoconstriction. The more we know about the physiological activity of these peptides, the easier could be the task of sorting out some lesser known mechanisms involved in the related diseases.

Thrombin induces polymerization of fibrinogen to fibrin by releasing peptide material of high negative charge, which apparently keeps the fibrinogen chains apart. Our studies with methylating agents now show that the reduction of negative charges on fibrinogen results in fibrinogen polymerization without the intervention of the enzyme thrombin. These results therefore suggest that the first step in the mechanism of clot formation is the removal of the negative repulsion at the ends of two of the fibrinogen chains without peptide material being necessarily simultaneously released.

Proposed Course of Project:

Bovine fibrinogen as a model for modified fibrinogen will be hydrolyzed with trypsin for various time intervals in an effort to release the fibrinopeptides. Their separation and isolation will also be undertaken.

The action of thrombin and trypsin on the modified fibrinogens will be investigated in an attempt to release modified fibrinopeptides. The separation and isolation of the modified fibrinopeptides will be attempted.

The physiological role of the native and modified peptides from fibrinogen will be further investigated.

Further chemical modification of specific functional groups in fibrinogen and the fibrinopeptides will be explored to gain greater insight into the relationship between structure, biological activity and enzymatic specificity.

Keyword Descriptors:

Fibrinogen, thrombin, modified fibrinogen, fibrinopeptide release, physiological activity, hemostasis, pulmonary hypertension, vasoconstriction, clot formation.

Publications:

1. Osbahr, A. J.: The Hemostatic Effect of Bovine Peptide-B from Fibrinogen: Thrombosis et Diathesis Haemorrhagica XXXII: 149-157, 1974.

2. Osbahr, A.J.: Structure-Activity Relationships of the Vasopressor Activity of Canine Peptide-A from Fibrinogen: Biochimica et Biophys. Acta 386, 373-381, 1975.

3. Osbahr, A. J.: The action of Peptide-B from Bovine Fibrinogen on ATPase Activity and Superprecipitation of Myosin B: American Journal of Physiology 228: 488-498, 1975.

Serial No. Z01 AM 31004 4 LBC
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Progress Report
July 1, 1974 through June 30, 1975

Project Title: The Elucidation of the Structure and Interactions of Biologically Important Macromolecules

Previous Serial Number: LBC-6

Principal Investigator: H. A. Saroff

Other Investigators: R. B. Simpson

Cooperating Investigator: Arthur A. Spector
Dept. of Biochemistry and Internal Medicine
University of Iowa
Iowa City, Iowa 52240

Cooperating Units: Clinical Endocrinology Branch, NIAMDD

Man Years:

Total: 2 1/2

Professional 2

Project Description:

Objectives:

The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accummulation of data on the state of the molecule with respect to its environment. Gross features of structures as well as fine detail may be observed by studies.

Methods:

In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecules, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements and other spectral techniques. For other properties of the protein molecules, we employ the ultra centrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies:

A. Raman spectra of fatty acids in aqueous solution were obtained to calculate the dimerization constant of these acids in water. (Simpson 2 years).

B. Oxidation of hemoglobin: The kinetics of oxidation of hemoglobin by hydroxylamine and by oxygen are being studied. It was found that in a closed vessel oxyhemoglobin solution is converted mainly to deoxyhemoglobin, rather than methemoglobin, indicating that there is an oxidation of something in the hemoglobin at a much faster rate than oxidation of the heme. This consumption of oxygen may account for literature reports of rates of methemoglobin formation that are much slower than we found in vessels open to the air. (Simpson, Saroff 1 1/2 years).

C. Action of hemoglobin: Binding data on hemoglobin allows for analysis of the cooperative effect in terms of the sites at which the energy of interaction is stored. Deoxyhemoglobin is a relaxed molecule (from the standpoint of the interaction energy) while oxyhemoglobin is raised to a higher potential energy level by an energetic increment equal to the interaction energy. The oxygenation data at different concentrations of protons and anions are being analyzed in conjunction with differential titration data for hemoglobin in order to quantitate the increment of interaction energy related to the pH dependent, or Bohr sites. (Saroff 4 years).

D. Cooperative effects: Data from the Clinical Endocrinology Branch have been analyzed in terms of cooperative systems. Negative cooperative effects were proposed for the binding of thyroxine to pre-albumin and cooperative effects deriving from association were proposed for the systems with hCG and hLH on the binding ANS (8-anilino-1-naphthalenesulfonic acid). (In conjunction with Robbins, Edelhock, Ferguson and Ingham 1 year).

Significance to Biomedical Research and the Programs of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the bio-medical significance of our findings derive from extrapolation to the biological system. Studies on the binding properties of protein systems related to certain hormones provide insight to possible modes of cooperative effects in hormone action.

Keyword Descriptors:

Proteins, structure, hemoglobin, cooperativity, fatty acids

Publications:

1. Ferguson, R. N., Edelhoch, H., Saroff, H. A. and Robbins, J.: Negative Cooperativity in the Binding of Thyroxine to Human Serum Albumin; Biochemistry, (1974), 14, 282.

2. Minton, A. P. and Saroff, H. A. : A General Formulation of the Free Energy of Interaction in Cooperative Ligand Binding; Application to Hemoglobin; Biophysical Chemistry (1974), 2, 296.

3. Simpson, R. B., Ashbrook, J. D., Santos, E. C. and Spector, A. A.: Partition of Fatty Acids; J. Lipid Res. (1974) 15, 415.

Serial No. Z01 AM 31,005-1 LBC
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Quantum Chemical Investigation of Biomolecules

Previous Serial No.: None

Principal Investigators: Dr. Janos J. Ladik, Visiting Scientist
Mr. Max Seel, Guest Worker

Cooperating Units: Dr. Albert Szent-Györgyi, Marine Biological Laboratory, Woods Hole, Mass.; Dr. Peter Otto and Mr. Alfred Karpfen, Theoretical Chemistry Institute, Technical University of Munich, Germany; Smith, Kline & French Laboratories, Philadelphia, Pa.

Man Years
Total: 2
Professional: 2

Project Description:

Electronic Structure of ATP

1) Complete Neglect of Differential Overlap (CNDO/2) calculations for free ATP and its bound form to the guanidinium residue of arginine and to the -CH_2-NH_2 group of lysine are in progress. In this way, we hope to get an insight into the mechanism of energy storage and release, respectively, when ATP is bound to actin.

Correlations Between Electronic Structure and Activity

2) Perturbation Configuration Interaction Using Localized Orbitals (PCILO) and CNDO/2 calculations have been performed for a series of substituted benzylamines which are potentially useful pharmacological agents for reducing epinephrine biosynthesis. A correlation has been established between the energy difference of the lowest empty and highest filled MO energies and the pharmacological activities. Applying the "leave out *n*" technique has shown that the found correlation most probably has a predictive power and can be used to construct the pharmacologically optimal compounds of the series.

Charge Transfer Complexes

3) In cooperation with Dr. Albert Szent-Györgyi, we have started *ab initio* calculations to investigate the charge transfer between model compounds

containing an -S- group of atoms and model compounds of the polypeptide chain (acceptors). These investigations have on the one hand the purpose to throw light on the possibility of charge transfer from -S- containing compounds (or protein side chains) to the polypeptide chain making it in this way a conductor. On the other hand, the used model compounds serve also as models for the TCNQ-TTF charge transfer system which is a metallic conductor with a possibility of superconductivity at 60°K.

Methods for Aperiodic Polymers

4) An *ab initio* "average matrix" method has been worked out to treat aperiodic polymers with not very different subunits. The application of the method for aperiodic DNA is planned. Further, a new procedure has been worked out to take into account the effect of a cluster of impurities on the band structure of an aperiodic polymer which can be applied again to periodic DNA and protein models.

Intermolecular Interactions

5) In copperation with the Institute of Theoretical Chemistry of the Technical University of Munich, further essential progress has been made in the mutually consistent field (MCF) theory of intermolecular interactions. In the Hartree-Fock case, the results reproduce those of the direct supermolecular approach. The development of the method for the case of correlated functions (to take into account dispersion effects) is in progress. It is planned to use this new method to study the interactions between biopolymer (DNA, protein) constituents and thus to determine theoretically their probable conformations.

DNA Energy Bands

6) Finally, again in cooperation with the Munich group, energy band structure calculations have been performed for the four DNA homopolynucleotides in the CNDO/2 and Modified Intermediate Neglect of Differential Overlap (MINDO/2) crystal orbital (CO) approximation in the presence of the inhomogeneous electric field caused by the PO_4^- - K^+ double layers of DNA. As it was expected, the electric field strongly influences the band structure causing a broadening of most bands.

Serial No. Z01 AM 31,006-6 LBC
1. Biophysical Chemistry
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Transglutaminase (Factor XIII) and tumor growth and actin as a nucleotide-binding protein.

Previous Serial No.: NIAMDD-LBC 6

Principal Investigators: K. Laki; G. Csako, Visiting Associate

Technical Assistance: E. F. Wilson

Cooperating Units: S. I. Chung, National Institute of Dental Research;
S. T. Yancey, National Cancer Institute

Man Years
Total: 2
Professional: 2 - 1/6

Project Description:

Objectives: In previous reports we have presented evidence indicating that transglutaminase (Factor XIII) plays a role in the growth and spread of tumors. This year we began to examine the possible relationship between tissue transglutaminase and tumor metastasis.

Muscle is an energy transducer converting the chemical energy of ATP into mechanical work. The protein, actin, is an essential element of the transducer; therefore, an attempt is made to reconstruct its three-dimensional structure.

Major Findings: Transglutaminase and tumor growth. It is now becoming realized worldwide that two enzymes, thrombin and transglutaminase (activated Factor XIII), must act on fibrinogen to produce a normal clot. If plasma transglutaminase is inoperative, a bleeding tendency arises which in children leads to fatal intracranial bleeding. In addition to functioning in hemostasis, Factor XIII is also involved in wound healing. We have reported animal experiments indicating that transglutaminase is also involved in tumor growth and metastasis. Our recent findings suggest that transglutaminase also plays a role in the metastasis of human tumors. It was found that the frequency of metastasis into the host organs correlates with the transglutaminase content of the organs.

The relationship between the frequency of metastasis and transglutaminase content of the human tissues may be expressed by the equation:

$$M = a.TG^b$$

Where M is the frequency of metastasis of breast tumor into the various organs, TG is the transglutaminase content of the tissues, and a and b are constants. According to our current studies, a similar relationship seems to exist in the metastasis of implanted tumors into the various organs of mice. The mechanism responsible for the correlation of transglutaminase and tumor metastasis is under investigation. (Laki, Yancey).

Actin as an ancient nucleotide-binding protein. Current theories depict the globular head of the myosin molecules as the site of force generation. As we have pointed out earlier, the globular portion of myosin shows great similarity to actin; thus, in our opinion, actin (F-actin) should be examined as a possible force-generating structure. And since actin under certain conditions cleaves ATP stepwise, it offers a simplified system for the study of how ATP is converted to mechanical work. Utilizing the known amino acid composition of actin, its resemblance to other nucleotide-binding proteins and also our experimental work which implicates an Arg(28), Lys(113), and perhaps Arg(39) residue in the binding of the γ-phosphate of ATP, we constructed a three-dimensional structure of the ATP binding domain of actin.

According to our analysis, the first three β-folds generating the nucleotide-binding β-pleated sheet in actin occurs at residues 42-48, 63-69, and 119-125. In analogy to adenylkinase, we arranged these three β-folds as βA, βC and βB. We propose that ATP bound to actin can shift to a low energy ATP while elevating the protein to an energized state through a tension developing in the β-pleated sheet or in a labile helix. For the cleavage of the low energy phosphate of ATP, we adopt the "pseudo rotation" mechanism of Westheimer and co-workers. (Laki, Ladik).

Keyword Descriptors: Transglutaminase, Factor XIII, metastasis, actin, nucleotide-binding proteins, energy transducer.

Significance to biomedical research and the program of the Institute: If the correlation between transglutaminase content of various tissues and frequency of metastasis becomes a general one, specific inhibitors yet to be discovered may be utilized to influence metastasis.

Proposed course of project: We are in the process of establishing the frequency of metastasis of different mouse tumors into the various organs of the mouse.

Publications:

1. Muszbek, L. and Laki, K.: Cleavage of actin by thrombin. Proc. Nat. Acad. Sci. USA 71: 2208-2211, 1974.

2. Laki, K. and Muszbek, L.: On the interaction of F-actin with fibrin. Biochim. Biophys. Acta 371: 519-525, 1974.

3. Laki, K.: An attempt to measure "disorder" in mental disorders. Perspect. Biol. Med. 18: No. 2, 157-161, 1975.

4. Muszbek, L., Gladner, J. A. and Laki, K.: The fragmentation of actin by thrombin. Isolation and characterization of the split products. Arch. Biochem. Biophys. 167: 99-103, 1975.

ANNUAL REPORT OF THE LABORATORY OF MOLECULAR BIOLOGY

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM AND DIGESTIVE DISEASES

The work of the Laboratory of Molecular Biology is directed to an explanation of biological processes at the molecular level and to an understanding of the physical and chemical foundations of biological processes. Areas of investigation in the laboratory include study of the mechanisms of replication of DNA and of genetic recombination, characterization of mammalian and bacterial viruses by genetic analysis, studies of the molecular basis of viral properties, studies of the mechanism of protein synthesis, physical and chemical studies of the structure and properties of polynucleotides and nucleic acids, characterization of protein-DNA interaction in chromatin, investigation of protein structure by X-ray diffraction, and theoretical studies of muscle contraction and of nerve membrane characteristics. Major advances in several of these areas have been made by members of the laboratory during the past year.

Chromatin Structure and Function.

Studies of chromatin structure and its relation to function, particularly to regulation of transcription in differentiated eukaryote cells has been continued. The studies of the gross structure of chromatin have involved a continuation of earlier investigations of the effects of the enzyme staphylococcal nuclease on chromatin, both purified and within nuclei. It is found that a limit digest of either chromatin or nuclei contains a series of eleven discrete double-stranded DNA fragments of size ranging between about 155 and 40 base pairs. The fragments can be obtained from chromatin preparations from a wide variety of sources, as well as from nucleoprotein complexes reconstituted from DNA and total histones. Furthermore, the source of the DNA has no effect upon the result. Thus, the pattern is determined by a specific conformation of the histones, rather than by any special nucleoside sequence within the DNA. Studies of reconstitutes in which only selected histone fractions are added confirms that no single histone species is sufficient to give rise to DNA fragments of discrete size, but that specific pairs of histones generate specific DNA fragments. These results can also be related to the "bead-and-string" model of chromatin proposed by other workers. It is possible to follow DNA digestion within nuclei from earliest times to limit digest, and to show that the DNA digestion pattern follows a single kinetic path through high molecular weight "multimer" structures to a "monomer" about 180 base pairs long, and then to the limit digest pattern described above. (Felsenfeld, Axel, Melchior, Sollner-Webb).

The structure of chromatin in the neighborhood of an actively transcribed gene has also been studied. It was shown that chromatin isolated from duck reticulocytes, which make globin messenger RNA, could serve as a template for E. coli RNA polymerase in vitro, leading to the synthesis of detectible globin messenger-like RNA. Little or no globin sequences are found in transcripts from erythrocyte chromatin, or from chromatin isolated from other tissues which do not manufacture globin message in vivo. Using staphylococcal nuclease and the polycation poly-D-lysine as probes, it is now possible to show that in reticulocyte chromatin a specific portion of the globin gene is covered by protein or otherwise made unreactive to chemical probes. No such region exists in the chromatin of erythrocyte chromatin. It is possible that this structural difference is related to the transcriptional activity. (Felsenfeld, Axel, Cedar)

The highly purified DNA ligase from rat liver nuclei was found to seal about half of the nicks in the DNA of reconstituted chromatin, the remainder being blocked specifically by histone binding. The histones do not block sealing of a specific half the nicks, implying histones do not bind to specific DNA sequences in the reconstituted chromatins. (Zimmerman, Levine)

Studies on DNA replication and recombination

1) Replication of colicin El plasmid DNA *in vitro*

We have established a soluble system to replicate closed-circular DNA molecules of colicin El plasmid (Col El). With the system, we further explored the mechanisms of *in vitro* replication of closed-circular DNA. When the plasmid DNA synthesized *in vivo* was labeled for a short time just before the termination of incubation, most of the labeled was found in open-circular molecules that had a nick or gap in their newly formed strand at or very close to the origin of replication. The open-circular molecule is converted to a closed-circular structure by further incubation. The addition of nicotinamide mononucleotide inhibited the conversion. These results indicate that replication of the circular molecules proceeds unindirectionally on both strands to completion of replication but two daughter molecules segregate from each other before formation of closed-circular molecules. The final sealing process by ligation or a reaction that immediately precedes the ligation is inhibited by nicotinamide mononucleotide. This system is the only known system that allows study of the mechanism of segregation of daughter molecules *in vitro*.

Cell extracts can support replication of Col El DNA added to the reaction mixture. *De novo* RNA synthesis is required for initiation of exogenous DNA as well as endogenous DNA. The RNA participates in DNA replication without translation into protein. Col El DNA replication depends entirely on bacterial function. This result is the first demonstration of complete replication of closed-circular double-stranded DNA added to a soluble system.

New electronmicroscopic method to determine the location of strand breaks in DNA molecules has been developed. Molecules with a strand break (nick or gap) were first treated with exonuclease III to partially digest DNA strands from the 3' end at the site of the break. When the sample of these molecules were examined under the electromicroscope the single-stranded region thus made appeared as a "bush". By using the endonuclease *Eco*Rl cleavage site as a reference site, the location of the nick can thus be determined. In addition to the form that is assumed to contain only deoxyribonucleotides linked by phosphodiester bonds, two other types of closed-circular Col El DNA has been described. One contains a protein called relaxation protein and other a sequence of RNA in the DNA strands. Removal of the protein or the RNA results in formation of strand-break in DNA molecules. We determined location of the strand-break by the above method. The results show that relaxation protein is located at or near the origin of replication and the RNA locates at random along the molecule. (Tomizawa, Sakakibara, Sugino, Bird).

2) Enzymatic joining of DNA strands and its role in genetic recombination

The "reverse" mutants of phage λ (λ rev) have been further characterized. The segment of host chromosomal DNA which is substituted in these phages has been identified as coming from the rac region. λ rev lysogenizes at rac, and hosts in which rac is deleted do not give rise to λ rev. In addition, the recombination nuclease coded by λ rev has been identified with the host enzyme exo VIII, whose chromosomal control locus sbcA is closely linked to rac.

There is evidence that other functions beside this nuclease have been incorporated in λ rev. We are now trying to study these genes by mutational methods. (Gellert, Mizuuchi, Wickner)

3) Novel recombination systems.

What appear to be semi-specific recombinational systems are being studied in two specialized transducing phages, λcam and P1-pro. A determinant for chloramphenicol-resistance, cam, initially detected on an RTF and since transferred to phage P1, may be acquired from P1 by phage λ. The acquisition of cam by λ is not dependent upon host RecA or λ Red functions (but might be determined by a P1 function). Two different locations of the cam DNA on λ have been demonstrated by electron microscopic heteroduplex mapping of two independently isolated λcam. The mobility of the cam DNA suggests that it has a mechanism for inserting itself into a number of unrelated genomes. Furthermore, the surprising loss of a variable portion of the cam DNA from λcam occurs at a high rate and is promoted by the λ Red function but not by the bacterial Rec function.

Induction of bacteria carrying the P1-pro prophage results in the production of lysates which transduce pro and the P1 gene 2 at high frequency. Unexpectedly, the pro DNA is able to integrate into the bacterial chromosome in at least 2 different places which are distant from the normal pro locus. This raises the question of whether the pro-gene 2 element possesses a system which allows it to integrate in different places. (Rosner)

Genetics and structure of the oncogenic virus, SV40.

By several criteria it has been shown that Chinese hamster lung cells transformed by ts A mutants of SV40 lose their transformed phenotype at the nonpermissive temperature. Thus the A function is required for maintenance of the transformed state. Since it is also known that A function is required for the initiation of viral DNA replication in permissive cells, it has been proposed that transformation by SV40 is a consequence of aberrant initiation of host DNA synthesis. A number of techniques are being employed to test this idea.

Psoralen is a compound which intercalates in DNA and which after near UV irridation produces cross links in the DNA. We have obtained conditions for cross-linking Chinese hamster lung cell DNA in vivo so that there is > 1 cross link per 10^6 daltons. When these cross-links are produced in cells which have been synchronized and are in G_1, and then allowed to enter S in the presence of H^3 thymidine, it is possible to obtain nascent DNA of chain length corresponding to the degree of cross-linking. On heating and rapidly cooling, the parental DNA snaps back to double stranded material, while the nascent chains remain single stranded. It is hoped in this manner to obtain the DNA corresponding to the initiation points of mammalian DNA replication. If these sequences can be obtained they will be compared with the corresponding sequences from transformed cells.

It has been observed that Chinese hamster lung cell DNA fragments during S phase and these observations have been extended to transformed and normal cells at permissive and nonpermissive temperatures.

One of the features of transformed cells not normally dealt with is the fact that such cells upon reaching very high cell densities slough from the monolayer and die. This observation suggested the possibility that all cells which are unable to enter a "resting" phase prior to the initiation of DNA synthesis might be particularly susceptible to depletion of the medium. Indeed, such a phenomenon in bacteria, namely, thymine-less death, might serve as a prototype. We have therefore examined the ability of various temperature sensitive mutants in DNA synthesis of E. coli to withstand sudden shifts from log phase growth at the permissive temperature to nonpermissive temperature. Mutants blocked in the elongation of nascent DNA, dnaE, dnaB, dnaF and lig^- mutants rapidly die in such shift experiments, whereas dnaA mutants blocked only in initiation of DNA synthesis are relatively stable. That there is nothing peculiar about the strain that carries the dnaA mutation has been shown by the fact that such a strain undergoes thymine-less death at the permissive but not at the nonpermissive temperature. (Martin, Chou, Brockman, Anderson, Avila, Persico-DiLauro)

Studies on polynucleotides

1) Poly 8-Bromoadenylic Acid, an all-Syn Polymer

Investigation of the important question of the effect of the uncommon syn conformation on properties of polynucleotides had been prevented until recently by lack of a method to synthesize the necessary polymers. We reported last year the first synthesis of an all-syn homopolymer, by using an oligo I primer in the presence of Mn^{++} and high concentration of polynucleotide phosphorylase. The primer was then removed from the purified polymer by selective cleavage with T_1 nuclease to give the homopolymer, poly 8-bromoadenylic acid. Previous theoretical predictions about syn polymers generally, and this one in particular, had held that they would be incapable of helix formation, or of single-strand stacking, and that the sugar conformation in such a polymer would be C2'endo. We have continued our investigation of poly (8BrA) and find most of its properties at variance with the predicted ones. Circular dichrosim measurements confirm our earlier ultraviolet studies indicating the polymer is strongly stacked. Recent magnetic resonance observations support our previous conclusion that the polymer exists as a rigid helix at room temperature. Observation of H1' spin-spin coupling at a temperature (80°) above the melting range of the poly (8BrA) helix suggest that the predominant sugar conformation is C3' endo rather than the predicted C2' endo. Poly (A) was used both as an external and internal control in these experiments by observing it separately and in mixtures with poly (8BrA). (Howard, Frazier, Miles)

2) Poly 2-Aminodenylic Acid

We had previously found that a 2-amino group introduced into poly(A) formed a third hydrogen bond in a Watson-Crick A·U pair markedly increasing the stability of the complex. Further study of interaction of poly($2NH_2A$) with poly(U) has shown that, in contrast to all other A, U systems, a 3 → 1 transition (simultaneous dissociation of all three strands) and a 2 → 3 transition (disproportionation) do not occur under any conditions. The three-stranded helix always undergoes a 3 → 2 transition (strandwise dissociation) even at very high ionic strength, again in contrast to other A, U systems. This large change in relative stabilities of two- and three-stranded helices results in a phase diagram relating Tm and cation concentration which is completely different from those of analogous A, U systems reported previously. The information so provided is invaluable for understanding the $2NH_2A$, U system and, more generally, in providing well-documented reference points for interpreting other polynucleotide interactions. (Howard, Frazier, Miles)

3) Structure of Triple Helices

The thermal stability of a new polynucleotide complex has been used to establish the hydrogen bonding structure of three-stranded $C \cdot G \cdot CH^+$ helices. In the Hoogsteen structure the $8NH_2$ group of $8NH_2GMP$ can form a third hydrogen bond to the CH^+ strand, but in the alternative structure the $8NH_2$ group can form no interbase hydrogen bonds. We observe for the new complex, $8NH_2GMP \cdot 2$ poly(C), a transition temperature of 80° under conditions in which the corresponding complex formed with 5'-GMP has a Tm of 20°. We conclude from this 60° elevation of transition temperature that a third hydrogen bond is formed by the $8NH_2$ group and that the structure must have Hoogsteen bonding. In order to be compatible with this structure in regular helices formed by U, C copolymers, A·2U bonding would also have to have a Hoogsteen structure. (Hattori, Frazier, Miles)

4) Poly 8-Aminoguanylic Acid. Synthesis of polymer and formation of helical complexes.

Poly ($8NH_2G$) was prepared by phosphorylation of the nucleoside, conversion to the diphosphate and polymerization with polynucleotide phosphorylase. Both of the latter steps proceeded with difficulty and in poor yield.

Poly($8NH_2G$) was found to have a novel helical structure of high stability, with no detectable melting below 100°. pH dependence, salt dependence, infrared spectra, circular dichroism, and ultraviolet spectra all indicate that the structure is a hemiprotonated one with a two-fold rotation axis. An unusual feature of this structure is that the three bonding sites lie along the "top" of the purine rings with bases offset, the imidazole rings opposite each other and the pyrimidine rings relatively exposed. The usual Watson-Crick bonding sites and all the usual hydrogen bonding protons are unpaired. Nevertheless, the stability of the hemiprotonated self-structure is so great that poly(C) does not react with poly($8NH_2G$) in neutral solution (see following sections).

The "acid" proton at N7 of the hemiprotonated helix is titrated cooperatively with a pK of 9.7 in 0.1 M Na^+. The fact that this value is 5 pK units above that of the monomer again reflects the very high stability of the helix. The form to which poly($8NH_2G$) is converted by this titration to pH 10 is not single-stranded but a second helix, which we think is probably four-stranded and similar in its bonding scheme to that proposed by Zimmerman et al. for poly(G) in neutral solution.

The highly stable G·G structure described above prevented interaction with poly(C) in neutral solution, even under annealing conditions which lead to G·C helices when poly(G) is used. By titrating poly($8NH_2G$) to pH 10 in the presence of poly(C), however, a two-stranded Watson-Crick helix was formed, and characterized by IR, UV and CD spectroscopy. The stability appears to be similar to the complex formed by poly(G) and poly(C), and the 8-amino group takes no part in hydrogen bonding. All attempts to form a three-stranded G·2C helix were unsuccessful, again being precluded by the hemiprotonated G·G structure. (Hattori, Frazier, Miles)

5) X-ray Diffraction Studies on Polyguanylic Acid

The Fiber diffraction and model building study of polyI and polyG indicate a four-stranded structure for both polymers. (Zimmerman)

Protein Structure

1) Structure and mechanism of proteases.

a) Continuing the refinement of γ chymotrypsin, the model has been subjected to several rounds of real space refinement procedures. Data are being collected to 1.8 A with a view to producing an accurate final model.

b) Acid protease from Rhizopus chinensis: Two new heavy atom derivatives have been discovered and the way has been prepared for a 3 A resolution map which will be computed shortly.

2) Immunoglobulin Structure.

a) Further model building studies have been carried out on the 3.1 A structure of the mouse phosphorylcholine-binding IgA McPC 603.

b) Comparisons of the structure with that of various other immunoglobulin chains has revealed close similarity and has demonstrated the invariant properties of the framework part of the molecule upon which the hypervariable surfaces may be draped in order to provide diversity of binding specificity for antigen.

c) Model building studies have extended this concept to explore the possibility of constructing binding sites for new antibodies from a knowledge of the amino acid sequence in conjunction with some chemical evidence. In particular, a model of the binding site of MOPC 315, a DNP-binding immunoglobulin, has been constructed.

d) Comparison of sequence data with the three-dimensional structure for the constant region of light chains has permitted an understanding of the sequence invariance of different parts of this chain. (Davies, Padlan, Navia, Silverton, Cohen).

e) A heavy chain (α) derived from a mouse myeloma IgA protein has been found to contain 100 fewer amino acid residues than other mouse α chains. The entire deletion appears to be in the C_H3 domain. (Robinson)

Protein Synthesis in Mammals

Previous investigations showed that polypeptide chain elongation factor 2, inactivated by diphtheria toxin and NAD^+, contains the ADP ribose moiety of NAD^+ in covalent linkage with an unidentified residue in the protein. The unidentified residue is a slightly basic α amino acid which gives a positive Pauly reaction and which does not appear to be any amino acid thus far described. The unknown amino acid has been isolated following acid hydrolysis of the protein, but not yet in sufficient quantity nor of sufficient purity to analyze by mass spectrometry and nuclear magnetic resonance.

A fraction of poly(A)-containing messenger RNA which codes for a product precipitable by anti-HPL sera has been isolated from term placentas. The precipitable material is closely related to HPL as analyzed by the pattern of tryptic peptides on column chromatography. Electrophoresis on SDS polyacrylamide gels shows that the synthesized material is slightly larger than authentic HPL. The possibility that the product is a natural precursor of HPL is under investigation. (Maxwell, Robinson, Cox, Tsai)

Calorimetric Investigations of Cellular Systems and Biological Macromolecules

The polymerization of hemoglobin S molecules into fibers and alignment of these fibers to form a crystalline phase (gelation) rigidifies the red cell and produces the distortion referred to as sickling. We have performed the first detailed kinetic and thermodynamic study of the gelation reaction. The thermally induced gelation of deoxyhemoglobin S exhibits unusual kinetic behavior. Prior to the onset of reaction there is a delay period which is followed by an autocatalytic progress curve. The delay period has a large apparent activation energy and is inversely proportional to about the 30th power of the deoxyhemoglobin S concentration.

The equilibrium solubility and heat of gelation of deoxyhemoglobin S has been determined over a wide temperature range. These results lead to an excellent first-order thermodynamic description of a gel as consisting of two phases in reversible equilibrium: a liquid phase containing monomeric hemoglobin and solvent and a more concentrated phase containing aligned fibers and solvent. The observed enthalpy changes are extremely small and positive indicating that little or no conformational change takes place in the protein upon gelation. There is a large negative heat capacity change associated with gelation, supporting the view that hydrophobic interactions are important in stabilizing the fiber phase.

We have developed a wide variety of techniques to study the gelation reaction; scanning microcalorimetry, optical birefringence, light scattering, water proton nmr linewidths and ultracentrifugation. Most recently, we have developed a technique to determine the rate of gelation and the solubility on a small amount of the same sample in a single tube. Since this method yields unequivocal kinetic and thermodynamic information it will be extremely useful in examining the effects of a wide variety of physiological parameters and possible therapeutic agents upon the rate of gelation and solubility properties of hemoglobin S. (Ross, Hofrichter, Eaton)

Energy Conversion in Biology, especially Muscle Contraction.

Work on this general topic during the past year can be subdivided into three classes:

a) Muscle contraction. The detailed formalism needed to treat any sliding filament model of muscle contraction has been extended to take into account several complications, among which are: multiple actin sites; double-headed myosin cross-bridges; interactions between cross-bridges; and stochastics and noise in muscle contraction. In addition to this work which is of a general nature, application has been made in detail to several two-state models of the Podolsky-Nolan type (modified to achieve self-consistency). Even though certainly too simple, these models produce essentially the correct mechanical and thermodynamic properties, including an efficiency of almost 40%. This indicates that the general approach includes the essential features involved in muscle contraction.

b) Free energy and fluctuations in steady-state systems. Steady-state systems are important in free energy transduction, for example in active transport and muscle contraction. This work examines several of the previously unknown but important properties of fluctuations in steady-state systems.

c) Hydrophobic interactions. A basic problem in all of molecular biology is the role of water structure and its relation to hydrophobic interactions. Several aspects of this topic have been investigated during the past year. (Hill, Chen, Ben-Naim, Podolsky, Eisenberg)

Ion Transport Across the Nerve Axon Membrane.

Our general objective in this work is to attempt to interpret experimental kinetic, steady-state, and noise data on K^+ (especially) and Na^+ transport across the nerve axon membrane in terms of a model in which the channels are protein complexes undergoing voltage-dependent conformation changes.

During the current period, we have pursued the noise problem somewhat further. Since noice involves the decay of spontaneous fluctuations, the kinetic parameters obtained from noise measurements are the same as those obtained from ordinary kinetic studies or chemical relaxation measurements. But, due to the fact that they can be carried out at steady-state or at equilibrium without applying any perturbation to the system as a relaxation method usually does, noise measurements may prove to be very useful in *in vivo* studies of biological systems.

In our new work, noise and fluctuations due to chemical reactions were considered for kinetic systems at a cycling *steady-state*. The derived noise power spectrum is expressed explicitly as a function of the kinetic rate constants so that numerical calculations can easily be carried out for an arbitrary cycling steady-state model. There are possible applications to optical pumping, photo adsorption-desorption reactions, active membrane transport, muscle contraction, etc. In each case, noise measurements would provide information about the kinetic scheme. (Hill, Chen)

Project No. Z01 AM 33000-09 LMB
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of functions involved in genetic recombination

Previous Serial Number: NIAMDD-LMB-4

Principal Investigator: Martin Gellert

Other Investigators: Kiyoshi Mizuuchi
Mary O'Dea
Raymond Devoret (Guest Worker)
Michiyo Mizuuchi (Guest Worker)

Man Years:

Total:	3 1/4
Professional:	2 3/4
Other:	1/2

Project Description:

1) Studies on "reverse" mutants of phage λ (M. M. Gottesman, K. Mizuuchi, M. Gellert). We previously reported that these mutants carry a substitution of DNA recombination genes derived from the E. coli chromosome. This substitution has now been further characterized. It comes from the region of the inducible genetic element rac, which is known from the work of B. Low to code for a recombination function. Rac^- strains do not give rise to λ reverse, and λ reverse integrates in the chromosome at, or very near, rac.

In addition to the general recombination function (der) by which λ reverse was first recognized, the phage seems to carry a second, site-specific, recombination function also derived from the host chromosome. This function is responsible for prophage integration.

Mutants of λ reverse have been isolated which express the Der function in the prophage state, and thus increase the survival of rec^- hosts following UV irradiation.

2) In vitro studies on the site-specific recombination system (Int) of phage λ (K. Mizuuchi, M. Mizuuchi).

a) A chemical assay for Int function has been developed. It measures intermolecular recombination between two λ DNA species: non-radioactive λ DNA which has been coupled to a filter by UV irradiation, and radioactive λ DNA circles in solution. The radioactivity bound to the filters is a measure of the reaction.

b) A selection procedure is being worked out for finding host mutants in which λ integration is defective. It involves a combination of galactose mutations and a promoter-constitutive λ phage which, upon integration, induces the galactose enzymes and kills the cell.

3) Studies on the mechanism of induction of phage λ (R. Devoret). It is now known that λ repressor is cleaved and inactivated upon UV irradiation of lysogenic cells. Efforts are under way to identify and study this process *in vitro*. The most promising approach involves the use of an *E. coli* mutant (*tif*) which spontaneously induces λ at temperatures above 40°.

Keywords: Genetic recombination / Escherichia coli / Bacteriophage lambda

Publications:

Gottesman, M. M., Gottesman, M. E., Gottesman, S. and Gellert, M.: Characterization of Bacteriophage λ Reverse as an *Escherichia coli* Phage Carrying a Unique Set of Host-Derived Recombination Functions. *J. Mol. Biol.* 88: 471-487, 1974.

Project No. Z01 AM 33001-06 LMB
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

Previous Serial Number: NIAMDD-LMB-5

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: Elizabeth A. Robinson
G. Stanley Cox
Miin-Rong Tsai

Cooperating Units: NIAMDD-CE (Saul Rosen and Bruce Weintraub)

Man Years:
Total: 3 1/2
Professional: 3 1/2

Project Description:

Our overall objective is to gain an understanding of the mechanisms and metabolic controls of protein synthesis in mammals. For the past few years we have worked toward obtaining a partial understanding of these processes through detailed knowledge of the soluble enzymes involved in polypeptide chain elongation. Work in this area is continuing, but we have now expanded our interest to include translation of specific messenger RNAs in crude systems.

Investigations of the chemical and enzymatic properties of Elongation factor 2, the soluble protein factor required for the translocation step in polypeptide chain elongation have continued. The tryptic peptide containing the site of the inactivation of the factor by NAD^+ and diphtheria toxin consists of 15 amino acid residues whose sequence is Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg. The ADP ribose is linked to the unknown residue, X. X is a weakly basic α amino acid which gives a positive Pauly reaction and which does not correspond to any amino acid commonly occurring in proteins nor to any of the known basic amino acids occurring less frequently. Attempts to isolate the unknown residue in sufficient amounts of sufficient purity to identify by mass spectrometry and nuclear magnetic resonance are in progress.

A study of the binding of guanosine nucleotides to EF-2, and the effects of ribosomes and Mg^{++} on the interaction has been completed. The data suggest that guanosine nucleotides modulate protein synthesis by affecting the binding of EF-2 to ribosomes and its release therefrom.

A project is underway to study the cell-free synthesis of the placental polypeptide hormones, HPL and HCG. This work is being done in collaboration with Drs. Saul Rosen and Bruce Weintraub, NIAMDD, CE.

Dr. G. Stanley Cox has isolated a fraction of poly(A)-containing RNA from term placenta which serves as a messenger RNA in a cell-free system from wheat germ. The product is precipitable by antiHPL serum and is closely related to HPL as analyzed by the pattern of labeled tryptic peptides on column chromatography. Electrophoresis on SDS polyacrylamide gels shows that the product is slightly larger than authentic HPL. The possibility that the product is a natural precursor of HPL is under investigation.

Keyword Descriptors: Protein synthesis, elongation factor 2, diphtheria toxin, guanosine nucleotides, rare amino acids, protein chemistry, placenta, messenger RNA, Human Placental Lactogen, wheat germ

Publications:

1) Robinson, Elizabeth A., Henriksen, Ole and Maxwell, Elizabeth S.: Elongation Factor 2: Amino Acid Sequence at the Site of Adenosine Diphosphate Ribosylation. J. Biol. Chem. 249: 5088-5093, 1974.

2) Maxwell, Elizabeth S., Robinson, Elizabeth A. and Henriksen, Ole. Elongation Factor 2: The Site of ADP-ribosylation. In "Proceedings of the Seminar on Poly(ADP-Ribose) and ADP-Ribosylation of Protein": Tomakomai, Hokkaido, Japan, Sept. 1974. J. Biochem. 77: 9P, 1975.

3) Henriksen, Ole, Robinson, Elizabeth A. and Maxwell, Elizabeth S.: Interaction of Guanosine Nucleotides with Elongation Factor 2: I. Equilibrium Dialysis Studies. J. Biol. Chem. 250: 720-724, 1975.

4) Henriksen, Ole, Robinson, Elizabeth A. and Maxwell, Elizabeth S.: Interaction of Guanosine Nucleotides with Elongation Factor 2: II. Effect of Ribosomes and Magnesium Ions on Guanosine Diphosphate and Guanosine Triphosphate Binding to the Enzyme. J. Biol. Chem. 250: 725-730, 1975.

Project No. Z01 AM 33002-02 LMB
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structural Studies on Mouse Myeloma Immunoglobulins

Previous Serial Number: NIAMDD-LMB-6

Principal Investigator: Elizabeth A. Robinson

Cooperating Units: Dr. Ettore Appella, NCI
Dr. David F. Smith, American Red Cross Blood Research Laboratory

Man Years:
Total: 1/3
Professional: 1/3

Project Description:

Objectives: Determination of the primary structure of two heavy chains from mouse IgA proteins, one of which is deleted.

Major Findings: The heavy chain (α) derived from a mouse myeloma IgA protein, which has an abnormally low molecular weight, was found to have a normal carbohydrate content but to have a deletion of 100 amino acids. Not only is the entire C_H3 domain missing but sequence differences in the hinge region have also been found.

Significance to NIAMDD Research: The elucidation of the structure of mouse myeloma immunoglobulins is pertinent to the understanding of the structure and specificity of human antibodies.

Proposed Course: To determine the complete amino acid sequence of the deleted mouse α chain.

Keyword Descriptors: Immunoglobulins, Myeloma

Publications:

Robinson, Elizabeth A., Smith, David F., and Appella, Ettore: Chemical Characterization of a Mouse Immunoglobulin A Heavy Chain with a 100-Residue Deletion. The Journal of Biological Chemistry: 249, 6605-6610, 1974.

Project No. Z01 AM 33051-03 LMB
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Enzymes from Mammalian Tissues which are Involved in the Repair or Replication of DNA

Previous Serial Number: NIAMDD-LMB-15

Principal Investigator: Steven B. Zimmerman

Other Investigator: Cora J. Levin

Man Years:
Total: 1 3/4
Professional: 3/4
Other: 1

Project Description:

Objectives: To isolate and characterize several enzymes from mammalian sources which act on DNA, including polynucleotide kinase and polynucleotide ligase, in order to characterize them generally and in particular to test their interaction with DNA in various regions and types of chromatin.

Methods: Standard techniques of biochemistry.

Major Findings: A highly purified DNA ligase from rat liver nuclei has been tested on DNA containing single-strand breaks ("nicks"); the DNA was present in several types of complexes which were chosen to serve as models for chromatin. These model systems included complexes of polylysine or histones with DNA as well as reconstituted chromatin preparations. In all cases, the limit of ligase sealing was measured as a function of the ratio of polypeptide or protein to DNA. With an excess of either polylysine or histones, the ligase is totally prevented from sealing nicks in the DNA. However, at ratios of histones to DNA similar to those occurring in chromatin, about half of the nicks are accessible to the ligase. In the reconstitution of chromatin, the proteins are dissociated from the DNA by exposure to high ionic strength either with or without urea. If such procedures are carried out in the presence of labeled nicked DNA, the proteins will redistribute over this ligase substrate as well. When the chromatin is reconstituted at protein to DNA ratios similar to those occurring in chromatin, once more only about half of the nicks are accessible to the ligase. Similar results were obtained with preparations reconstituted with either rat liver or duck reticulocyte chromatin. The rate of ligase action has also been measured on a variety of the complexes. While the rate falls as the DNA is increasingly covered with polylysine or histones, this is largely or entirely due to the decrease in concentration of sealable sites. At saturating concentrations of these DNA complexes, the original rate on uncovered DNA is approached.

When reconstituted chromatin is treated with an excess of DNA ligase, about half of the nicks are sealed. If this partially sealed preparation is taken through a second cycle of reconstitution, a large fraction of the previously unsealed nicks are now sealed upon exposure to the DNA ligase. The changes in the fraction of accessible nicks are in quantitative agreement with a random binding model in which all DNA sequences are equally suitable for binding of histones and rule out the binding of histones to a specific subset of DNA sequences in these model chromatin preparations.

Significance to Bio-Medical Research and the Program of the Institute: The ligase and kinase, as well as other enzymes, are thought to function in repair and replication of DNA. Damaged regions in DNA have been implicated in a variety of disease states as well as in aging and differentiation. A large proportion of the DNA is covered with proteins in chromatin and the present project is aimed at assessing the ability of these enzymes to work on such a substrate. It is possible the enzymes may also serve as a probe of chromatin structure, itself a controversial subject.

Proposed Course: We have partially purified the polynucleotide kinase of rat liver and are characterizing this activity. It has clearly different specificity than the only other known polynucleotide kinase. Also we will continue studies of the subcellular distribution of the DNA ligase of this organ and attempt the isolation of a mitochondrial enzyme.

Keyword Descriptors: DNA, enzyme, kinase, ligase

Publications:

Zimmerman, S. B. and Levin C. J.: A Deoxyribonucleic Acid Ligase from Nuclei of Rat Liver: Purification and Properties. J. of Biol. Chem. 250: 149-155, 1975.

Zimmerman, S. B. and Levin, C. J.: Do Histones Bind to a Specific Group of DNA Sequences in Chromatin? A Test Based on DNA Ligase Action on Reconstituted Chromatin. BBRC 62: 357-361, 1975.

Zimmerman, S. B. and Levin, C. J.: DNA Ligase Activity on Chromatin and Its Analogues: Rejoining of DNA Strands in Polylysine-DNA Complexes and in Reconstituted Chromatins†. Biochemistry 14: 1671-1676, 1975.

Project No. Z01 AM 33052-03 LMB
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: X-ray Diffraction Studies on Polyguanylic Acid

Previous Serial Number: NIAMDD-LMB-17

Principal Investigator: Steven B. Zimmerman

Other Investigators: David R. Davies
Gerson H. Cohen

Man Years:
Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: To obtain structural information on poly G through x-ray fiber diffraction techniques.

Methods: Standard techniques of biochemistry and x-ray diffraction.

Major Findings: Further detailed model building studies on an interactive computer system lend strong support to our contention that poly G and poly I have the same four-stranded structure.

At acidic pH values, poly G adopts a different structure. While the diffraction patterns are consistent with a double-helical acid form, detailed studies must await higher quality diffraction patterns.

Significance to Bio-Medical Research and the Program of the Institute: Both normal and diseased states of tissues are controlled to an impressive extent by polynucleotides. Structural studies on polynucleotides may offer new insights into the functioning of these molecules. The current work constitutes the first relatively strong evidence for the existence of new type of secondary structure, namely a four-stranded structure.

Proposed Course: The final results of this project are being prepared for publication.

Keyword Descriptors: poly G, poly I, RNA, x-ray, fiber

Publications:

1) Zimmerman, S. B., Cohen, G. H. and Davies, D. R.: X-ray Fiber Diffraction and Model-building Study of Polyguanylic Acid and Polyionosinic Acid. J. Mol. Biol. 92: 181-192, 1975.

2) Sasisekharan, V., Zimmerman, S. and Davies, D. R.: The Structure of Helical 5'-Guanosine Monophosphate. J. Mol. Biol. 92: 171-179, 1975.

Project No. Z01 AM 33053-03 LMB
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Kinetics and Thermodynamics of Hemoglobin S Gelation

Previous Serial Number: NIAMDD-LMB-14

Principal Investigators: Philip D. Ross
James Hofrichter (NIAMDD-LCP)
William A. Eaton (NIAMDD-LCP)

Man Years:
Total: 2.7
Professional: 2.7

Project Description:

Objectives: To gain a basic physical-chemical understanding of the sickling phenomenon and to interpret the behavior of potentially therapeutic additives in terms of their effects upon the kinetics and thermodynamics of gelation.

Methods: Differential scanning and isothermal calorimetry. Optical birefringence and light scattering. Ultracentrifugation and measurement of water proton line widths.

Major Findings:

The rigidification and distortion of the red cell in sickle cell disease arises from the polymerization of hemoglobin S molecules into fibers and alignment of these fibers to form a crystalline phase (gelation).

We have performed the first detailed kinetic and thermodynamic study of the gelation reaction.

The thermally induced gelation of deoxyhemoglobin S as observed quantitatively by calorimetry or qualitatively by optical techniques exhibits unusual kinetic behavior. Prior to the onset of reaction there is a delay period which is followed by an autocatalytic progress curve. The delay period has a large apparent activation energy and is inversely proportional to about the 30th power of the deoxyhemoglobin S concentration.

We have developed a kinetic mechanism accounting for the marked concentration, temperature and time dependence of this reaction.

The equilibrium solubility and heat of gelation of deoxyhemoglobin S has been determined over a wide temperature range. These results lead to an excellent first-order thermodynamic description of a gel as consisting of two phases in reversible equilibrium: a liquid phase containing monomeric hemoglobin and solvent and a more concentrated phase containing aligned fibers and solvent. The observed enthalpy changes are extremely small and positive indicating that little or no conformational change takes place in the protein upon gelation. There is a large negative heat capacity change associated with gelation, as is characteristic of interacting protein systems.

We have developed a technique to determine the rate of gelation and the solubility on a small amount of the same sample in a single tube. Since this method yields unequivocal kinetic and thermodynamic information it will be extremely useful in examining the effects of a wide variety of physiological parameters and possible therapuetic agents upon the rate of gelation and upon the solubility properties of hemoglobin S.

Significance to Biomedical Research and the Program of the Institute:
The results and implications of our kinetic study bring a new point of view toward understanding the pathophysiology of sickle cell disease.

The mechanism and kinetic theory developed in this study may be applicable to other macromolecular assembly processes.

The methods so far developed will be useful in examination and interpretation of the effects of potential therapuetic agents upon the sickling process.

Proposed Course of Project: The fundamental physical-chemical experiments and theoretical work will be continued. The effects of additives will be studied. These studies will be extended to the sickling process in erythrocytes.

Keywords: Hemoglobin S gelation, Calorimetry, Macromolecular Assembly, Thermodynamics, Kinetics, Pathophysiology, Nucleation theory, Sickle cell therapy

Publications:

Ross, P. D. and Goldberg, R. N.: A Scanning Microcalorimeter for Thermally Induced Transitions in Solution. Thermochimica Acta 10: 143-151, 1974.

Hofrichter, J., Ross, P. D. and Eaton, W. A.: Kinetics and Mechanism of Deoxyhemoglobin S Gelation: A New Approach to Understanding Sickle Cell Disease. Proc. Natl. Acad. Sci. USA 71: 4864-4868, 1974.

Hofrichter, J., Ross, P. D. and Eaton, W. A.: Kinetic and Thermodynamic Investigation of Deoxyhemoglobin S Gelation. In Hercules, J. I., Schechter, A. N., Eaton, W. A. and Jackson, R. E. (Eds): *Proceedings of the First National Symposium on Sickle Cell Disease*. Washington, D.C., U.S. Government Printing Office, 1974, pp. 43-45.

Ross, P. D., Hofrichter, J. and Eaton, W. A.: Calorimetric and Optical Characterization of Sickle Cell Hemoglobin Gelation. *J. Mol. Biol.*, in press.

Ross, P. D., Scruggs, R. L. and Manning, G. S.: Measurements and Interpretation of the Conductance of DNA in Simple Salt Solutions. *Biopolymers*, in press.

Serial No. Z01 AM 34000-11 LMB
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure of Single Strand Polynucleotides in Solution

Previous Serial Number: 12

Principal Investigator: Gary Felsenfeld

Other Investigators: Bethel Stannard

Man Years:

Total: 1/4
Professional: 1/4
Others: 0

Project Description:

Objectives: To elucidate the structure of single strand polynucleotides in solution.

Methods Employed: Theoretical analysis, light scattering, viscometry, spectrophotometry, sedimentation velocity, sedimentation equilibrium.

Major Findings: We have concluded our studies of single stranded synthetic polynucleotides in solution. These began with an investigation of polyadenylic acid (poly A) under ideal solvent conditions, and continued to a study of poly U, apurinic acid, and finally poly A at very low temperature. The studies show that at all temperatures the backbone of a polynucleotide confers considerable rigidity upon the molecule. This behavior has been accounted for in a semi-theoretical analysis which introduces approximate potential energies of rotation about the backbone bands. At low temperatures, poly A shows additional rigidity from base stacking. Our studies make it possible to analyze the entire temperature-dependent stacking reaction in poly A, and to understand the effects of stacking and intrinsic backbone rigidity upon conformation.

Significance to Bio-medical Research and the Program of the Institute:

This work completes our investigation of the nature of single strand polynucleotide structures in solution, the relative roles of base stacking, backbone rotational restrictions, and solvent interactions. The conformation of such molecules is a major determinant in their chemical behavior, and this behavior plays a dominant role in the reactivity of naturally occurring RNA, such as messenger RNA, viral RNA, ribosomal and transfer RNA, under natural conditions in the living cell.

Keyword Descriptors: Polynucleotides, conformation.

Publications: Stannard, Bethel J. and Felsenfeld, Gary: The Conformation of Polyriboadenylic Acid at Low Temperature and Neutral pH: A Single-stranded Rodlike Structure. Biopolymers 14: 299-307 (1975).

Serial No. Z01 AM 34001-10 LMB
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chromatin Structure and Function

Previous Serial Number: 13

Principal Investigator: Gary Felsenfeld

Other Investigators: William B. Melchior, Jr., Georgianna S. Sandeen,
Barbara Sollner-Webb, Patrick L. Williamson,
Rafael D. Camerini-Otero and Francois X. Wilhelm

Man Years:

Total: 5
Professional: 5
Others: 0

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation. DNA-RNA hybridization methods are used to detect presence of specific polynucleotide sequences. Gel electrophoresis methods are used for measuring DNA and protein size, for examining nucleoprotein preparations, and for detecting chemically cross-linked proteins within chromatin.

Major Findings: We have examined in greater detail the nature of the digestion products resulting when the enzyme staphylococcal nuclease attacks chromatin. We find that the limit digest consists of a series of well-defined discrete fragments of double-stranded DNA ranging in size from about 160 to 40 base pairs. Similar fragments are generated when histones and DNA from any source (eukaryote or prokaryote) is reconstituted with total histone. The fragments are generated by specific interaction between histone complexes and the DNA in which well defined protein segments are in contact with the

DNA surface. By reconstituting chromatin with only a subset of histones, we have been able to identify the band pattern as arising from specific groups of histones. One discrete set of bands arises from the arginine-rich pair (H3 + H4) while another set arises from the interaction between arginine-rich histone and the slightly lysine-rich pair (H2A + H2B). Four other bands arise from lysine-rich histone interactions with all the other histones.

We have also followed the nuclease digestion process from earliest times of digestion to the limit, using both nuclei and chromatin as substrate. We confirm work in other labs showing a "subunit" structure within chromatin, and are able to show that the higher order oligomer nucleoprotein subunits released from nuclei under mild digestion conditions are true precursors of a nucleoprotein monomer containing DNA 185 base pairs in length. Further digestion then leads to the small fragments described above.

We are continuing work with specific transcriptional properties of chromatin, and now hope to relate the structural features described above to the known ability of chromatin preparations from duck reticulocytes to serve as templates *in vivo* for the transcription of the globin genes. We have shown that in active reticulocyte chromatin a specific portion of the globin gene is inaccessible to polylysine, quite probably because it is covered by protein.

Significance to Bio-medical Research and the Program of the Institute:

Chromatin is the nucleoprotein complex that appears to contain the chemical information for cellular differentiation. Understanding of the organization of chromatin proteins on DNA and the way in which they affect transcription and other DNA functions in higher organisms will lead to an understanding of how cells differentiate, and how they maintain this differentiated state, in which only a relatively small fraction of the total gene population of the cell is expressed. This is a central problem of cell biology.

Proposed Course of Project: We propose to examine the abundance of the globin gene in the various nucleoprotein fragments we have isolated. We are examining protein-protein contacts in nucleoprotein monomer units, and studying the chemistry of the intact subunits. We hope to identify the region of the globin gene which is preferentially covered by protein in active reticulocyte chromatin. We also hope to begin looking for and purifying those proteins specifically responsible for maintaining active globin gene transcription.

Keyword Descriptors: Chromatin, histones, differentiation, transcription, hemoglobin, nucleoprotein.

Publications:

Felsenfeld, G., Axel, R., Cedar, H. and Sollner-Webb, B.: The Specific Template Activity of Chromatin. In Ciba Foundation Symposium 28: *The Structure and Function of Chromatin*. The Netherlands, Associated Scientific Publishers, 1975, pp. 29-57.

Axel, R., Melchior, Jr., W., Sollner-Webb, B. and Felsenfeld, G.: Specific Sites of Interaction Between Histones and DNA in Chromatin. Proc. Nat. Acad. Sci. U.S.A. 71: 4101-4105, 1974.

Axel, Richard, Cedar, Howard and Felsenfeld, Gary: The Structure of the Globin Genes in Chromatin. Biochem., in press.

Sollner-Webb, Barbara and Felsenfeld, Gary: A Comparison of the Digestion of Nuclei and Chromatin by Staphylococcal Nuclease. Biochem., in press.

Sollner-Webb, Barbara and Felsenfeld, Gary: Protein Interaction with DNA in Chromatin. Florida Molecular Biology Colloquium, in press.

Serial No.: Z01 AM 34002-11 LMB
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: X-ray Diffraction Investigation of Proteolytic Enzymes

Previous Serial Number: 10

Principal Investigators: David R. Davies, Easwara Subramanian,
Gerson H. Cohen and Enid W. Silverton

Man Years:

Total: 3
Professional: 3
Others: 0

Project Description:

Objective: The determination of the 3-dimensional structure of proteolytic enzymes, in particular, the structure of γ-chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: X-ray diffraction analysis.

Major Findings: 1. The refinement of the coordinates of tosyl γ-chymotrypsin at 2.7 A resolution has been continued and is now almost complete. Data have been collected to extend the resolution to 2 A and are being extended farther to 1.8 A.

2. Two new heavy atom derivatives of the acid protease from Rhizopus chinensis have been discovered and data are currently being collected in order to provide a 3 A map of the protein. The use of these new derivatives has permitted the calculation of a 5.5 A map which very clearly defines the outlines of the molecule and redefines its dimensions as 60 x 35 x 45 Å. One of the outstanding features of the map has been tentatively identified as a "β-barrel" of the kind previously observed in chymotrypsin and in the superoxide dismutage.

Significance to Bio-medical Research and the Program of the Institute: Although the general mechanism of action of the serine proteases is fairly well understood, there is still some disagreement concerning the precise details. The refinement of γ-chymotrypsin at high resolution and different pH's should help resolve this problem.

The acid protease is one of a large class of enzymes that include pepsin, rennin and the cathepsins. It has sequence homology with other members of the class and the elucidation of its structure should ultimately reveal the mechanism of action of these proteins.

Proposed Course of Project: To calculate and interpret a 3.0 Å map. Subsequently to collect higher resolution data and extend the structure to high resolution.

Keyword Descriptors: Chymotrypsin, acid protease, Rhizopus chinensis, proteolysis, enzyme, three-dimensional structure.

Serial No.: Z01 AM 34003-07 LMB
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Crystal Structure Investigation of Antigen Antibody Interaction

Previous Serial Number: 9

Principal Investigators: David R. Davies, Eduardo A. Padlan, Enid W. Silverton and Manuel A. Navia

Cooperating Units: Michael Potter, NCI and Stuart Rudikoff, NCI

May Years:

Total: 3
Professional: 3
Others: 0

Project Description:

Objectives: A comparative investigation of the crystal structure of the Fab fragment of immunoglobulin molecules that bind strongly to several different antigens. In this way, it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with the heavy atom isomorphous replacement method. Model building. Computer comparisons of structure.

Major Findings: The last year has been a period of consolidation and refinement for the crystal structure of the mouse myeloma protein McPC 603. Detailed models have been constructed and refined and coordinates have been measured. These have been used to make comparisons with other Fab and Bence-Jones structures. It has been established quantitatively that the domain structure consists of a more or less invariant framework plus the hypervariable loops that are directly involved in antigen binding.

A model has been constructed of the binding site of another mouse myeloma protein, MOPC 315, that binds DNP ligands. This model was based on the known structure of McPC 603 plus the amino acid sequence of MOPC 315. This exercise demonstrated the necessity for more structural information before such models can be truly constructed with confidence.

The pattern of sequence homologies in the C_L region of mouse and human κ and λ chains has been related to the three-dimensional structure.

Proposed Course: 1) The crystal structure of McPC 603 is being extended to incorporate more heavy atoms and higher resolution.

2) The structure of J539, a galactan binding protein, is being examined.
3) Attempts are being made to crystallize intact mouse myeloma proteins and their Fc fragments.

Significance to Bio-medical Research and the Program of the Institutes: An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Keyword Descriptors: Immunoglobulin, mouse, myeloma, phosphorylcholine, three-dimensional structure, Fab, antigen, binding site.

Publications:

Padlan, E.A., Segal, D.M., Cohen, G.H., Davies, D.R., Rudikoff, S. and Potter, M.: The Three-Dimensional Structure of the Antigen Binding Site of McPC 603 Protein. In Sercarz, E.E., Williamson, A.R. and Cox, C.F. (Eds.): The Immune System. New York, Academic Press, 1974, pp. 7-14.

Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M. and Davies, D.R.: The Three-Dimensional Structure of a Phosphorylcholine-Binding Mouse Mouse Immunoglobulin Fab and the Nature of the Antigen Binding Site. Proc. Nat. Acad. Sci. U.S.A. 71: 4298-4302, 1974.

Segal, D.M., Padlan, E.A., Cohen, G.H., Silverton, E.W., Davies, D.R., Rudikoff, S. and Potter, M.: The Structure of McPC 603 Fab and Its Hapten Complex. In Brent, L. and Holborow, J. (Eds.): Progress in Immunology II, Vol. I: Immunochemical Aspects. Amsterdam, North Holland Publishing Company, 1974, pp. 93-102.

Padlan, E.A., Segal, D.M., Cohen, G.H., Davies, D.R., Rudikoff, S. and Potter, M.: Structure of a Phosphorycholine-Binding Mouse Myeloma Fab. Transplant. Proc., 1975, in press.

Padlan, E.A. and Davies, D.R.: Variability of Three-Dimensional Structure in Immunoglobulins. Proc. Nat. Acad. Sci. U.S.A. 72: 819-823, 1975.

Labaw, L.W., Padlan, E.A., Segal, D.M. and Davies, D.R.: An EM Study of Phosphorylcholine-Binding Fab' Immunoglobulin Fragment Crystals. J. Ultrastruct. Res., in press.

Davies, D.R., Padlan, E.A. and Segal, D.M.: Three-Dimensional Structure of Immunoglobulins. Ann. Rev. Biochem., in press.

Davies, D.R., Padlan, E.A. and Segal, D.M.: Immunoglobulin Structures at High Resolution. Contemporary Topics in Molecular Immunology, Vol. 4, in press.

Kabat, E.A., Padlan, E.A. and Davies, D.R.: Evolutionary and Structural Influences on the Light Chain Constants (C_L) Region of Human and Mouse Immunoglobulins. Proc. Nat. Acad. Sci. U.S.A., in press.

Serial No. Z01 AM 35000-11 LMB
1. Laboratory of Molecular Biology
2. Section on Organic Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Chemical and Structural Investigations of Nucleic Acids and Related Substances

Previous Serial Number: NIAMDD-LMB-11

Principal Investigator: Dr. H. Todd Miles

Other Investigators: Dr. M. Hattori, Dr. F. B. Howard, and Mr. J. Frazier

Man Years:

Total:	4-1/2
Professional:	3-1/2
Other:	1-1/2

Project Description:

Structure of Triple Helices. The thermal stability of a new polynucleotide complex has been used to establish the hydrogen bonding structure of three-stranded $G \cdot G \cdot CH^+$ helices. In the Hoogsteen structure the $8NH_2$ group of $8NH_2GMP$ can form a third hydrogen bond to the CH^+ strand, but in the alternative structure the $8NH_2$ group can form no interbase hydrogen bonds. We observe for the new complex, $8NH_2GMP \cdot 2$ poly(C), a transition temperature of 80° under conditions in which the corresponding complex formed with 5'-GMP has a Tm of 20°. We conclude from this 60° elevation of transition temperature that a third hydrogen bond is formed by the $8NH_2$ group and that the structure must have Hoogsteen bonding. In order to be compatible with this structure in regular helices formed by U, C copolymers, $A \cdot 2U$ bonding would also have to have a Hoogsteen structure.

In both the monomer-polymer helix described here and the polymer-polymer helix described above the 8-amino group plays a dominant role, yet the contrast between the two systems is almost complete. In the monomer-polymer case the high stability imparted by a third hydrogen bond to the $8NH_2$ group completely prevents formation of a 1:1 complex, which would lack this third strand. The low stability of the hemiprotonated $G \cdot G$ monomer complex prevents it from interfering with $G \cdot 2C$ helix formation. The former system, on the other hand, with the addition of a protonated poly(C) third strand cannot compete with the high stability of the hemiprotonated $G \cdot G$ structure. As a result, only the double helix is formed.

8-Bromoadenylic Acid, an all-Syn Polymer. Investigation of the important question of the effect of the uncommon syn conformation on properties of polynucleotides had been prevented until recently by lack of a method to synthesize the necessary polymers. We reported last year the first synthesis of an all-syn homopolymer, by using an oligo I primer in the presence of Mn# and high concentration of polynucleotide phosphorylase. The primer was then removed from the purified polymer by selective cleavage with T_1 nuclease to give the homopolymer, poly 8-bromoadenylic acid. Previous theoretical predictions about syn polymers generally and this one in particular had held that they would be incapable of helix formation, or of single-strand stacking, and that the sugar conformation in such a polymer would be C2'endo. We have continued our investigation of poly(8BrA) and find most of its properties at variance with the predicted ones. Circular dichroism measurements confirm our earlier ultraviolet studies indicating the polymer is strongly stacked. Recent magnetic resonance observations support our previous conclusion that the polymer exists as a rigid helix at room temperature. Observation of H1' spin-spin coupling at a temperature (80°) above the melting range of the poly(8BrA) helix suggest that the predominant sugar conformation is C3' endo rather than the predicted C2' endo. Poly(A) was used both as an external and internal control in these experiments by observing it separately and in mixtures with poly(8BrA).

Poly 2-Aminodenylic Acid. We had previously found that a 2-amino group introduced into poly(A) formed a third hydrogen bond in a Watson-Crick A·U pair markedly increasing the stability of the complex. Further study of interaction of poly($2NH_2A$) with poly(U) has shown that, in contrast to all other A, U systems, a 3 → 1 transition (simultaneous dissociation of all three strands) and a 2 → 3 transition (disproportionation) do not occur under any conditions. The three-stranded helix always undergoes a 3 → 2 transition (strandwise dissociation) even at very high ionic strength, again in contrast to other A, U systems. This large change in relative stabilities of two- and three-stranded helices results in a phase diagram relating Tm and cation concentration which is completely different from those of analogous A, U systems reported previously. The information so provided is invaluable for understanding the $2NH_2A$, U system and, more generally, in providing well-documented reference points for interpreting other polynucleotide interactions.

Proximity Effects on Helix Stability. It is known that Br and Me groups in pyrimidine position 5 increase the stability of the helices formed by polynucleotides containing them. In trying to understand the origin of these stabilizing effects we have asked the question whether the effect requires close proximity of the modified bases to each other or whether it can be exerted equally well upon an adjacent unmodified base. We had previously examined copolymers of C and BrC, finding that the elevation of Tm was linear in BrC content in poly(I)·poly(C,Br) helices. There was no detectable proximity effect. Previous workers had reported a non-linear dependence for the U,MeU and U,BrU systems. We have reexamined these systems as well as C,MeC copolymers in detail and find that for 2 → 1 transitions of r(C,MeC)·r(I) and of r(U,MeU)·rA [r(A)·r(BrU) does not undergo a 2 → 1 transition] and for 3 → 1 transitions of r(A)·2r(U,MeU) and r(A)·2r(U,BrU) the elevation of Tm is linear in content of altered pyrimidine. There is therefore no proximity effect for the indicated transitions in these polynucleotide systems.

Poly 8-Aminoguanylic Acid. In ordered structures of nucleic acids the amino groups perform a vital function in forming the hydrogen bonds upon which specificity of pairing depends. We have investigated the role of amino groups in determining the geometry and stability of base pairing interactions by introducing new amino groups into the purine rings of both monomers and polynucleotides. Hydrogen bonds formed by the 8-amino group in the present case cause striking changes in properties of ordered structures, described in this and the following sections.

Synthesis of the nucleotide 5'-$8NH_2GMP$, not previously reported, proceded without great difficulty by the action of $POCl_3$ on the nucleoside in trimethylphosphate at 0° (procedure of Yoshikawa and Kato). Formation of the diphosphate by the method of Moffat and Khorana, however, was (uncharacteristically) very difficult and proceded in low yield because of low solubility of the nucleotide in solvents favorable for the reaction.

Polymerization of the diphosphate also proved to be difficult with polynucleotide phosphorylase from either E. coli or M. luteus. Oligo A primer increased the yield with $8NH_2GMP$, though it did not do so in parallel experiments with GDP. A low but acceptable yield of poly($8NH_2G$) was obtained in an unprimed synthesis with the M. luteus enzyme at 50° in the presence of Mn^{++}. No reaction occurred at 37°.

Poly($8NH_2G$) was found to have a novel helical structure of high stability, with no detectable melting below 100°. pH dependence, salt dependence, infrared spectra, circular dichroism, and ultraviolet spectra all indicate that the structure is a hemiprotonated one with a two-fold rotation axis. An unusual feature of this structure is that the three bonding sites lie along the "top" of the purine rings with bases offset, the imidazole rings opposite each other and the pyrimidine rings relatively exposed. The usual Watson-Crick bonding sites and all the usual hydrogen bonding protons are unpaired. Nevertheless, the stability of the hemiprotonated self-structure is so great that poly(C) does not react with poly($8NH_2G$) in neutral solution (see following sections).

The "acid" proton at N7 of the hemiprotonated helix is titrated cooperatively with a pK of 9.7 in 0.1 M Na^+. The fact that this value is 5 pK units above that of the monomer again reflects the very high stability of the helix. The form to which poly($8NH_2G$) is converted by this titration to pH 10 is not single-stranded but a second helix, which we think is probably four-stranded and similar in its bonding scheme to that proposed by Zimmerman et al. for poly(G) in neutral solution. A further ordered structure was detected as a minor component in alkaline solution, but because of the unfavorable conditions it was not characterized in detail.

The highly stable G·G structure described above prevented interaction with poly(C) in neutral solution, even under annealing conditions which lead to G·C helices when poly(G) is used. By titrating poly($8NH_2G$) to pH 10 in the presence of poly(C), however, a two-stranded Watson-Crick helix was formed, and characterized by IR, UV and CD spectroscopy. The stability appears to be similar to the complex formed by poly(G) and poly(C), and the 8-amino group takes no part in hydrogen bonding. All attempts to form a three-stranded G·2C helix were unsuccessful, again being precluded by the hemiprotonated G·G structure.

Keyword Descriptors:

Pyrimidine, helices, polynucleotides, copolymers, proximity effect, transitions, monomers, polymerization, titration, spectroscopy.

Honors and Awards:

None

Publications:

Howard, F. B., Frazier, J and Miles, H. T.: Poly 8-Bromoadenylic Acid: Synthesis and Characterization of an All-Syn Polynucleotide, J. Biol. Chem., 1975, in press.

Hattori, M., Frazier, J. and Miles, H. T.: Ordered Forms of 5'-8-Aminoguanylic Acid, Biopolymers, 1975, in press.

Serial No. Z01 AM 35050-04 LMB
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Replication, Recombination and Repair of Microbial DNA

Previous Serial Number: NIAMDD-LMB-7

Principal Investigator: J. Tomizawa

Other Investigators: Y. Sugino
A. C. Satterthwait
R. E. Bird
H. Ohmori

Man Years:

Total:	2-3/12
Professional:	2-3/12
Others:	0

Project Description:

We have established a soluble system to replicate closed-circular DNA molecules of colicin E1 plasmid (Col E1). In the previous report, we described that cell extracts prepared from E. coli carrying the plasmid can support replication of enodogenous Col E1 DNA. The major products of replication are completely replicated molecules and a class of replicative intermediates. The intermediate contains a DNA fragment(s) that have a sedimentation constant of approximately 6 S. Synthesis of the intermediates is sensitive to rifampicin suggesting involvement of RNA polymerase functions for DNA replication. Once replication is initiated, further replication to its completion is not inhibited by rifampicin. On the other hand, it is blocked by the addition of 10% glycerol and 2 mM spermidine. Electronmicroscopic examination of these products showed that replication is initiated at a specific region of a molecule and proceeds unidirectionally. We continued to refine the system and explored the mechanisms of in vitro replication of closed-circular DNA. When the plasmid DNA synthesized in vivo was labeled for a short time just before the termination of incubation, most of the labeled DNA was found in open-circular molecules that had a nick or gap in their newly formed strand at or very close to the origin of replication. The open-circular molecule is converted to a closed-circular structure by further incubation. The addition of nicotinamide mononucleotide inhibited the conversion. These results indicate that replication of the circular molecules proceeds unidirectionally on both strands to completion of replication but two daughter molecules segregate from each other before formation of closed-circular molecules. The final sealing process by ligation or a reaction that immediately preceeds the ligation is inhibited by nicotinamide mononucleotide. This system is the only known system that allows study of the mechanism of segregation of daughter molecules *in vitro*.

Cell extracts can support replication of Col El DNA added to the reaction mixture. *De novo* RNA synthesis is required for initiation of exogenous DNA as well as endogenous DNA. The RNA participates in DNA replication without translation into protein. Col El DNA replication depends entirely on bacterial function. This result is the first demonstration of complete replication of closed-circular double-stranded DNA added to a soluble system.

New electronmicroscopic method to determine the location of strand breaks in DNA molecules has been developed. Molecules with a strand break (nick or gap) were first treated with exonuclease III to partially digest DNA strands from the 3' end at the site of the break. When the sample of these molecules prepared by the basic protein film technique were examined under the electronmicroscope the single-stranded region thus made appeared as a "bush". By using the endonuclease *Eco*Rl cleavage site as a reference site, the location of the nick can thus be determined. In addition to the form that is assumed to contain only deoxyribonucleotides linked by phosphodiester bonds, two other types of closed-circular Col El DNA has been described. One contains a protein called relaxation protein and the other a sequence of RNA in the DNA strands. Removal of the protein or the RNA results in formation of strand-break in DNA molecules. We determined location of the strand-break by the above method. The results show that relaxation protein is located at or near the origin of replication and the RNA locates at random along the molecule.

Keyword Descriptors:

Colicin El, plasmid, DNA replication, electronmicroscopy, RNA primer

Honors and Awards:

None

Publications:

Tomizawa, J., Sakakibara, Y. and Kakefuda, T.: Replication of Colicin El Plasmid DNA in Cell Extracts. Origin and Direction of Replication. *Proc. Nat. Acad. Sci. USA* 71: 2260-2264, 1974.

Sakakibara, Y. and Tomizawa, J.: Termination Point of Replication of Colicin El Plasmid DNA in Cell Extracts. *Proc. Nat. Acad. Sci. USA* 71: 4935-4939, 1974.

Tomizawa, J., Sakakibara, Y. and Kakefuda, T.: Replication of Colicin El Plasmid DNA Added to Cell Extracts, *Proc. Nat. Acad. Sci. USA* 72: 1050-1054, 1975.

Sugino, Y., Tomizawa, J. and Kakefuda, T.: Location of non-DNA Components of Closed Circular Colicin El Plasmid DNA. Nature 253: 652-654, 1975.

Project No. Z01 AM 36002-01 LMB
1. Laboratory of Molecular Biology
2. Section on Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Novel Recombination Systems

Previous Serial Number: None

Principal Investigator: J. L. Rosner (12/12)

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

What appear to be semi-specific recombinational systems are being studied in two specialized transducing phages, λcam and P1-pro. A determinant for chloramphenicol-resistance, cam, initially detected on an RTF and since transferred to phage P1, may be acquired from P1 by phage λ. The acquisition of cam by λ is not dependent upon host recA or λ Red functions (but might be determined by a P1 function). Two different locations of the cam DNA on λ have been demonstrated by electron microscopic heteroduplex mapping of two independently isolated λcam. The mobility of the cam DNA suggests that it has a mechanism for inserting itself into a number of unrelated genomes. Furthermore, the surprising loss of a variable portion of the cam DNA from λcam occurs at a high rate and is promoted by th λ Red function but not by the bacterial Rec function.

Induction of bacteria carrying the P1-pro prophage results in the production of lysates which transduce pro and the P1 gene 2 at high frequency. Unexpectedly, the pro DNA is able to integrate into the bacterial chromosome in at least 2 different places which are distant from the normal pro locus. This raises the question of whether the pro-gene 2 element possesses a system which allows it to integrate in different places.

Keyword Descriptors:

Chloramphenicol-resistance; specialized transduction; recombination; bacteriophage λ; bacteriophage P1.

Rosner, J. L.: Specialized transduction of *pro* genes by coliphage P1: Structure of a partly diploid P1-*pro* prophage. *Virology*, in press.

Serial No. Z01 AM 36051-07 LMB
1. Laboratory of Molecular Biology
2. Section on Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Genetics and structure of the oncogenic virus, SV40

Previous Serial Number: NIAMDD-LMB-1

Principal Investigator: Robert G. Martin (12/12)

Other Investigators: Jesus Avila (12/12); Jeffrey L. Anderson (12/12); William Brockman (12/12); Maria DiLauro (9/12)

Cooperating Units: Dr. Janice Chou, CH:LBS

Man Years:

Total:	4-9/12
Professional:	4-9/12
Other:	0

Project Description:

Objective: To analyze the functions of SV40 employing conditional lethal mutants. Special emphasis will be placed on the early A gene product which is required for the maintenance of transformation.

Major Findings: (a) The early function of SV40 encoded by the A gene has been demonstrated to be required for the maintenance of transformation. Transformation properties shown to be temperature sensitive in either Chinese hamster lung cells or mouse brain cells transformed by ts A mutant include: 1) ability to overgrow a monolayer; 2) ability to clone in soft agar; 3) ability to clone with high efficiency on plastic; 4) ability to grow in low serum; 5) rate of growth in low serum; 6) ability to form dense multilayered clones; 7) ability to achieve high saturation densities and 8) tendency for cells to slough and die at very high densities. Not temperature sensitive are: 1) transport of 2-deoxyglucose; 2) T-antigen production or 3) chromosome number (Anderson and Martin).

b) It has been noted that SV40 transformed cells die at very high cell densities. To test if all cells die when DNA synthesis cannot be completed we have investigated the death of E. coli mutants which are temperature sensitive for DNA synthesis. Cells which are blocked in the initiator but not elongation of nascent DNA strands are relatively resistant to incubation at the nonpermissive temperature. Cells which are synchronized prior to the initiation of DNA synthesis by amino acid starvation do not die at the nonpermissive temperature. These results are analogous to what is found in thymineless death. (Anderson and Martin)

c) The DNA of nontransformed cells becomes fragmented early in S phase and then returns to high molecular weight. The DNA of transformed cells remains fragmented throughout the cell cycle (Avila and Martin).

d) SV40 DNA synthesis can be followed in nuclear extracts. Essentially all of the synthesis however involves the completion of replicative intermediates, little if any new initiation occurs. Attempts are underway to reduce the extent of completion without interferring with initiation by cross-linking the SV40 DNA with psoralen. (DiLauro and Martin)

Keyword Descriptors:

SV40, transformation, cancer, DNA synthesis, psoralen, death.

Publications:

Chou, J. Y. and Martin, R. G.: Products of complementation between temperature-sensitive mutants of simian virus 40. J. Virol. 15, 127-136, 1975.

Chou, J. Y. and Martin, R. G.: DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. 15, 145-150, 1975.

Martin, R. G. and Chou, J. Y.: Simain virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. 15, 599-612, 1975.

Martin, R. G., Chou, J. Y., Avila, J. and Saral, R.: The semiautonomous replicon: A molecular model for the oncogenicity of SV40. Cold Spr. Harb. Lab. Quant. Biology, Vol. 39, 17-24, 1975.

Martin, R. G. and Anderson, J. L.: Death and transformation. Gatlinburg Conf. on Radiation Carcinogensis. In press, 1975.

Serial No. Z01 AM 36101-02 LMB
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Energy conversion in biology, especially muscle contraction

Previous Serial Number: NIAMDD-LMB-18

Principal Investigator: Dr. T. L. Hill (12/12)

Other Investigators: Dr. Y. Chen (6/12); Dr. A. Ben-Naim (12/12)

Cooperating Units: Lab of Phys. Biol., NIAMDD (Dr. R. Podolsky);
Lab of Biochem., NHLI (Dr. E. Eisenberg)

Man Years:

Total:	2-1/2
Professional:	2-1/2
Other:	0

Project Description:

Work on this general topic during the past year can be subdivided into three classes:

(a) Muscle contraction (see the first three publications, below). The detailed formalism needed to treat any sliding filament model of muscle contraction has been extended to take into account several complications, among which are: multiple actin sites; double-headed myosin cross-bridges; interactions between cross-bridges; and stochastics and noise in muscle contraction. In addition to this work which is of a general nature, application has been made in detail to several two-state models of the Podolsky-Nolan type (modified to achieve self-consistency). Even though certainly too simple, these models produce essentially the correct mechanical and thermodynamic properties, including an efficiency of almost 40%. This indicates that the general approach includes the essential features involved in muscle contraction.

(b) Free energy and fluctuations in steady-state systems (see the next three publications). Steady-state systems are important in free energy transduction, for example in active transport and muscle contraction. This work examines several of the previously unknown but important properties of fluctuations in steady-state systems.

(c) Hydrophobic interactions (see the last four publications). A basic problem in all of molecular biology is the role of water structure and its relation to hydrophobic interactions. Several aspects of this topic have been investigated during the past year.

Keyword Descriptors:

Muscle contraction, active transport, free energy transduction.

Publications:

Hill, T. L.: Theoretical formalism for the sliding filament model of contraction of striated muscle (Part II). Prog. Biophys. Mol. Biol. 29 (in press). 1975.

Hill, T. L., Eisenberg, E., Chen, Y., and Podolsky, R. J.: Some self-consistent two-state sliding filament models of muscle contraction. Biophys. J. 15, 335 (1975).

Chen, Yi-der: Phenomenological coefficients and general proof of reciprocal relation for a certain class of sliding filament model of muscle contraction. J. Theore. Biol. (in press) 1975.

Hill, T. L.: Free energy and the kinetics of biochemical diagrams, including active transport. Biochemistry (in press) 1975.

Hill, T. L.: Stochastics of cycle completions (fluxes) in biochemical kinetic diagrams. Proc. Nat. Acad. Sci. (in press) 1975.

Saito, Nobuhiko: Fluctuations in chemical reactions around the steady states. J. Chem. Phys. 61 3644 (1974).

Ben-Naim, A.: Solubility, hydrophobic interaction and structural changes in the solvent. J. Electrochem. Soc. (in press) 1975.

Ben-Naim, A.: Hydrophobic interaction in water-p-dioxan systems. J. Phys. Chem. (in press) 1975.

Ben-Naim, A.: Structure breaking and structure promoting processes in aqueous solutions. J. Phys. Chem. (in press) 1975.

Ben-Naim, A.: Hydrophobic interaction and structural changes in the solvent. Biopolymers (in press) 1975.

Serial No. Z01 AM 36102-04 LMB
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Statistical thermodynamics of polynucleotide - complementary monomer interactions

Previous Serial Number:

Principal Investigator: Dr. T. L. Hill

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Others: 0

Project Description:

The objective here is: (a) to clarify the thermodynamic procedures which should be used in comparing optical, calorimetric, and binding measurements on these systems; and (b) to develope and use, as a prerequisite, a theory of aggregation of monomers in solution.

Extensive work has already been done under both (a) and (b). Applications in the future to particular systems await the completion of joint experimental work by Dr. Philip Ross and Dr. Thomas Schleich (Univ. of Calif., Santa Cruz).

Keyword Descriptors:

Polynucleotides, monomer binding, thermodynamics, aggregation.

Serial No. Z01 AM 36103-04 LMB
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ion transport across the nerve axon membrane

Previous Serial Number: NIAMDD-LMB-20

Principal Investigator: Dr. Terrell L. Hill (0)

Other Investigators: Dr. Y. Chen (6/12)

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Others:	0

Project Description:

Our general objective in this work is to attempt to interpret experimental kinetic, steady-state, and noise data on K^+ (especially) and Na^+ transport across the nerve axon membrane in terms of a model in which the channels are protein complexes undergoing voltage-dependent conformation changes.

During the current period, we have pursued the noise problem somewhat further. Since noise involves the decay of spontaneous fluctuations, the kinetic parameters obtained from noise measurements are the same as those obtained from ordinary kinetic studies or chemical relaxation measurements. But, due to the fact that they can be carried out at steady-state or at equilibrium without applying any perturbation to the system as a relaxation method usually does, noise measurements may prove to be very useful in *in vivo* studies of biological systems.

In our new work, noise and fluctuations due to chemical reactions were considered for kinetic systems at a cycling *steady-state*. The derived noise power spectrum is expressed explicitly as a function of the kinetic rate constants so that numerical calculations can easily be carried out for an arbitrary cycling steady-state model. There are possible applications to optical pumping, photo adsorption-desorption reactions, active membrane transport, muscle contraction, etc. In each case, noise measurements would provide information about the kinetic scheme.

Keyword Descriptors:

ion transport, nerve membrane, current noise.

Publications:

Chen, Yi-der: Fluctuations and noise in kinetic systems III. Cycling steady-state models. J. Theoret. Biol. (in press) 1975.

Saito, N.: Localized oscillations in neural networks I. General considerations with nonlinear differential equations. J. Theor. Biol. 47, 421-437, 1974.

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

A mathematical model describing the diffusion and consumption of oxygen in the presence of myoglobin has been completed. A computer program has been written incorporating membranes, impermeable to myoglobin, in the diffusion path. The solution of the diffusion equation in terms of the kinetic constants of the oxygenation reaction, the diffusion coefficients of the species and intermembrane distances is obtained. Thus the effect of each of these parameters on the facilitation of oxygen diffusion by myoglobin can be studied. The available histological data on striated muscle indicate that the presence of membranes does not markedly interfere with facilitation (Dr. J. Gonzalez-Fernandez and Mrs. S. Atta).

A comprehensive chapter, on core conductor theory and cable properties of neurons, has been completed for the new Handbook of the Nervous System (in preparation by the American Physiological Society). In addition to its expository content and a unified treatment and overview of earlier results this chapter contains a substantial number of new and previously unpublished theoretical results. Studies of synaptic input to single dendritic branches reported last year (in collaboration with Dr. John Rinzel) have been pursued with special reference to implications for dendritic spines. These results reported at several symposia last year are being prepared for publication (Dr. Wilfrid Rall).

In a geriatric health study (conducted by Dr. Max Woodbury, Duke University) large samples of subjects are rated on a number of factors relating to physical and emotional well being. Cluster analysis identifies a number of typical states and transitions between them which can be studied by the use of directed graphs. A property called "parallelism" is defined for pairs of transitions and it is demanded that the property be transitive (edges parallel to a given edge are parallel to each other). In certain cases this formalism leads to anamolous results in that two or more parallel edges converge on a given node. An algorithm has been devised and programmed to detect this latter property in a graph of any complexity. Graph-theoretic studies on a characterization of the property are being carried out (Ms. R. B. Marimont, Associate member MRB).

Analytical and numerical work on potentials produced by population of cells has continued. In some important cases, notably including the olfactory bulb, cells are distributed in a spherical shell punctured at one point by the exit path of the output axons. Analytical solutions have been obtained for the potential generated by a spherical shell with arbitrary puncture. These solutions enable the complete potential distribution to be computed and compared to the predictions of various qualitative models which have been developed. It has been found that single cells and closed shells represent limiting cases of the intermediate populations. The intermediate population potentials are generally closer to that of closed shells than that of a single cell (Dr. Maurice Klee).

Previous work on the theoretical analysis of renal function has been extended in several directions. Finite difference equations describing salt and water movement in a model of the mammalian kidney have been solved numerically by an extension of the Newton-Raphson method reported previously (Proc. Nat. Acad. Sci. U.S.A., 71:1618-1622, 1974). The method permits both steady state and transient solutions. For the first time it has been possible to simulate behavior of the whole kidney as a function of hydrostatic pressures in renal artery, vein, and pelvis, protein concentration in arterial blood, and phenomenological equations describing transport of salt and water across nephron and capillary walls. With the model it has been possible to compute concentrations and hydrostatic pressures in the various nephron segments and in cortical and medullary capillaries and interstitium. In a general way calculations from the model have met intuitive expectations. In addition, they have re-emphasized the critical dependence of renal function on the hydraulic and solute permeabilities of glomerular, post glomerular, and medullary capillaries. These studies lend further support to our thesis that the functional unit of the kidney is not the individual nephron, but a nephrovascular unit consisting of a group of nephrons and their tightly coupled vasculature. Improvement of numerical methods for solving the difference equations describing various models continues as a major project. During the year a variety of partitioning schemes have been developed for solving models piecewise. With these schemes and existing computer technology it appears that it is going to be possible to develop some highly realistic multinephron, multisolute models of the kidney. (John L. Stephenson, Ray Mejia, NHLI, Associate Members, R. P. Tewarson, SUNY, Stony Brook, N.Y.)

Two survey chapters including recent past and especially current research on neuronal integration and impulse conduction along an axon are being completed. One is for Lectures on Mathematics in Life Sciences, published by the American Mathematical Society, the other for Studies in Mathematical Biology, published by the Mathematical Association of America. In collaboration with Dr. W. Rall, the solutions for single branch input to a neuron have been applied to the functional role of dendritic spines. A study of a simplified version of the FitzHugh conduction equation has been carried out and the traveling wave solutions obtained explicitly. The results indicate that repetitive firing produces a periodic train the propagation velocity of which is a function of firing frequency. This finding warrants further numerical exploration and possible suggestion of experiments designed to explore this aspect of conduction. The multiplicity of traveling wave solutions exhibited by this equation has led to speculation regarding the stability of these solutions. An explicit stability analysis has been carried out to indicate which of these solutions should not be observed (Dr. John Rinzel).

A review chapter on Monte Carlo methods for cooperative Ising models has been prepared which will appear in Cooperative Phenomena in Biology, Cambridge University Press. A numerical method is being devised which will allow very fine spatial resolution in three-dimensional reconstruction from x-rays and at the same time require relatively few angles and hence less x-ray exposure in human subjects. The method is much faster than existing methods but still allows reconstruction of finer detail. Work on three-dimensional reconstruction of macromolecules from electron micrographs has continued. A set of interactive programs for reconstruction and display of asymmetric molecules have been written and tested on chick ribosome dimers. The programs are applicable to any set of tilted micrographs (Dr. Richard Gordon).

Serial No. Z01 AM 13,000-02 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mathematics of kinetics and reaction-transport systems

Previous Serial Number: NIAMDD, ODIR, MRB-1

Principal Investigator: John Z. Hearon

Man Years:

Total:	1.3
Professional:	1
Others:	.3

Project Description:

Objectives: The objective of this research remain substantially as stated in previous reports.

Major Findings: In addition to consultative and collaborative efforts, work during the past year centered on problems of linear algebra of importance for the study of biological systems.

Lumping in Linear Systems.

The theory of lumping in linear systems is now complete. The necessary and sufficient conditions for k compartments in an n-compartment system to obey an $m<n$ order differential system have been obtained in terms of invariant subspaces of certain submatrices of the system matrix. Cast in this form their validity is clear for *any* linear system. In particular we do not require the usual physical restrictions of tracer analysis, diagonability of the matrix etc. The great variety of instances of lumping reported in the literature can all now be subsumed under one general theorem.

Control Theory in Linear Systems.

In many disparate contexts it is of considerable interest to know if given the system $\dot{x} = Ax + Bu$, $x(0) = x^0$ an input vector u can be chosen such that at $t=t_1$, $x(t_1)$ is a prescribed vector. If so the control vector u steers the system to an arbitrary state, specified in advance, at time t_1.

Given this possibility it is a further question whether a control vector of the form $u=cf(t)$, c a constant vector will suffice. If it will we can reduce control of the system to the scalar function $f(t)$. A set of lemmas have been proved which relate the degree of the minimum polynomial of range B, the rank of the controllability matrix and the degree of the minimum polynomial of A. These result in a simple theorem giving the necessary and sufficient conditions for reduction to scalar control.

The Leslie Population Matrix

Of considerable interest in population studies is the Leslie matrix which governs the difference equations of growth when the population is divided into age groups for each of which is specified a fertility rate and a survival rate. It has been shown that a simple similarity transformation puts the Leslie matrix in companion form. It is then possible to write explicitly the characteristic polynomial, characteristic vectors and principal vectors in the case of multiple roots. Bounds for the dominant root, which governs the asymptotic behavior, are given.

Generalized Matrix Norm

Vector and matrix norms are essential in the study of asymptotic behavior, convergence of iterative methods and error analysis for numerical techniques. We have discovered a generalized matrix norm induced by two vector norms. The multiplicative character of this norm is governed, strictly, by its consistency with one of the vector norms. The generalized norm includes most of the popular norms and many not previously studied.

Invariant Inner Product

In a variety of problems it is essential to know that a solution vector x of a system $Ax=y$, while not unique, does uniquely determine a given function $f(x)$. Least square estimation, three dimensional reconstruction from projections and determining generalized inverses with certain minimal properties are a few of the many problems in which this question arises. In all of these and many more, the function f is a linear functional. This broad class of cases is settled by the following theorem: The inner product (w,x) where x is a solution of $Ax=y$, is uniquely determined if and only if w is a vector in the range of A^*, the adjoint of A.

Keyword Descriptors: Linear systems, control theory, matrix theory, linear algebra.

Serial No. Z01 AM 13,001-02 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology

Previous Serial Number: NIAMDD, ODIR, MRB-2

Principal Investigator: Wilfrid Rall

Other Investigators: John Rinzel
Maurice Klee

Man Years:

Total:	1.3
Professional:	1.0
Others:	.3

Project Description:

Over the past several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models. This group of models provides a theory that can account for many different sequences of events in a dendritic neuron, and in certain populations of such neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with the results of neurophysiological experiments and neuroanatomical observations. Also, insights have been obtained, which have led to new hypotheses, new interpretations, and to the design of new experiments.

1. The completion of a comprehensive chapter, entitled, "Core conductor theory and cable properties of neurons", for the new Handbook of the Nervous System, Volume I, Cellular Biology of Neurons, (now in preparation for The American Physiological Society). In addition to perspective on earlier results, this chapter incorporates a number of new (unpublished) theoretical developments.

2. (With Dr. Rinzel, formerly of the Laboratory of Applied Studies, DCRT; now MRB). Two mathematical papers concerned with current injection or synaptic input to a single dendritic branch have appeared in the Biophysical Journal: one in 1973 and one in 1974. The implications relating to dendri-

tic spines have been presented at several symposia and will be prepared for publication during the next fiscal year.

3. (With Dr. Klee). Presentation and analysis of the characteristics of electric fields generated by activity in a single neuron and in open and closed cortical arrangements of neuron populations. Building upon my earlier research, Dr. Klee has carried out extensive computations, with special attention to the preparation of illustrations designed to be useful to experimental neurophysiologists. Considerable effort has been devoted to organizing both old and new theoretical results in a comprehensive context, probably as a set of three related theoretical papers.

Keyword Descriptors: Theoretical neuron models, mathematical models, cable theory of neurons, neuronal dendritic branching, neuronal input resistance and response functions, electric fields generated by single neurons and by populations of neurons.

Advisory Council:

USA National Committee for IBRO, National Research Council; 1972-1976.

Publications:

Rinzel, J. and Rall, W.: Transient response in a dendritic neuron model for current injected at one branch. _Biophys. J._ _14_: 759-790, 1974.

Goldstein, S. S. and Rall, W.: Changes of action potential shape and velocity for changing core conductor geometry. _Biophys. J._ _14_: 731-757, 1974.

Serial No. Z01 AM 13,002-03 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mathematical description of the transport-chemical reaction kinetics of substances in the blood-capillary-tissue complex

Previous Serial Number: NIAMDD, ODIR, MRB-3

Principal Investigator: Jose M. Gonzalez-Fernandez

Other Investigators: Susie E. Atta

Man Years:

Total:	2.3
Professional:	2
Others:	.3

Project Description:

A mathematical model describing the diffusion-consumption of oxygen in tissues in the presence of myoglobin was completed. A computer program was written incorporating the presence of membranes not permeable to myoglobin on the diffusion path. A relation between the coefficients of the chemical kinetics $O_2+Mb \rightleftarrows O_2Mb$, the diffusion coefficients of the species O_2 and O_2Mb and the distance between the membranes was obtained; this relation expresses the effect of these parameters on the facilitation. By using the available histological data for striated muscles it appears that the presence of membranes in the muscle does not interfere substantially with the potential facilitation.

Work is in progress to incorporate the kinetics coefficients of the tetramer hemoglobin in its combination with oxygen in the description of its facilitated diffusion.

A manuscript has been written for an algorithm to improve the rate of convergence of the iterative solution for a system of algebraic equations.

Keyword Descriptors: Myoglobin, hemoglobin, oxygen diffusion, facilitated diffusion, skeletal muscle, linear equations, overrelaxation.

Serial No. Z01 AM 13,003-02 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Applications of electric field theory to electrophysiology

Previous Serial Number: NIAMDD, ODIR, MRB-4

Principal Investigator: Maurice Klee

Man Years:

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objectives: To obtain an understanding of the electrical behavior of nerve and muscle in terms of three dimensional electric field theory.

Methods: Numerical and analytic techniques are used to calculate the electric fields within and surrounding both single cells and groups of cells.

Major Findings and Significance: During the past year in collaboration with Dr. Wilfrid Rall this project has concentrated on the problem of the potentials produced by populations of cells. In the past, various investigators have developed qualitative models to interpret the experimental recordings obtained from pools of neurons. Among these was the model proposed by Dr. Rall and Dr. Gordon Shephard of Yale University to explain the population potentials of the rabbit olfactory bulb. The cells of this preparation are distributed in spherical shells punctured at one end by the exit path of the mitral (output) cell axons. To interpret the recordings from this preparation, Drs. Rall and Shephard adopted a two step procedure: first they calculated the potentials one would expect from a completely closed spherical shell; then they shunted these potentials by a parallel path representing the possibility for current flow through the puncture. This process worked well for the olfactory bulb. Unfortunately, it was misunderstood by a number of investigators and some criticism appeared in the literature.

To better understanding these population potentials this project developed analytic solutions for the potentials generated by spherical shells of arbitrary puncture. Using these solutions complete potential distributions were calculated and then compared with the predictions of the various qualitative models. The major findings were: 1) The potentials of the single cell and the closed spherical shell can be separated out as limiting cases bounding the behavior of the intermediate populations. 2) In most cases, the intermediate population potentials are closer to those of the closed sphere than of the single cell. 3) The Rall and Shephard model does predict the qualitative changes of the closed sphere potentials produced by puncture.

During the coming year further calculations for more general conditions will be carried out in order to develop an improved qualitative model. This approach is mandated by the complex geometry of the nervous system which limits the applicability of analytic solutions and thus calls for a sound qualitative model capable of broad application.

Keyword Descriptors: Extracellular fields, field potentials, population potentials, potential divider theory, closed and open fields.

Publications:

Klee, M. and Plonsey, R.: Extracellular stimulation of a cell having a non-uniform membrane. IEEE Trans. Biomed. Engn. 21: 452-460, 1974.

Serial No. Z01 AM 13,004-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mathematical description of cellular neuroelectric signal transmission

Previous Serial Number: None

Principal Investigator: John Rinzel

Man Years:

Total:	.4
Professional:	.4
Others:	0

Project Description:

Objectives: To develop and analyze mathematical models to obtain biophysical understanding, of integration in a dendritic neuron and impulse conduction along a nerve axon. To expose the mathematical structure for a class of models by exploiting simple, yet reasonable, equations.

Methods: Mathematical formulations of physiologically interesting problems for core conductors involve systems of ordinary and partial differential equations. Analytical and numerical techniques are used to qualitatively describe and explicitly obtain their solutions.

Major Findings: Mathematical results and biophysical implications of recent past and present research efforts are included in two survey chapters which are being completed for publication. The first, "Simple model equations for active nerve conduction and passive neuronal integration", is for Lectures on Mathematics in the Life Sciences, vol. 8, to be published by the American Mathematical Society. The second, "Integration and propagation of neuroelectric signals", will appear in Studies in Mathematical Biology being prepared by the Mathematical Association of America. Some of these results and others, obtained from ongoing research, are summarized below.

Passive Dendritic Integration.

In a series of recent papers with Dr. Wilfrid Rall analytic solutions were obtained which describe time independent and transient response for input to a single branch location of an idealized neuron model. A paper which summarizes the transient solutions and compares response characteristics for input to different locations has appeared in Federation Proceedings (April, 1975). Solutions from these papers have also been applied in a theoretical examination of the functional role of synaptic dendritic spines. A manuscript on this additional application will be prepared.

Active Axonal Conduction.

A simplified version of Dr. Richard FitzHugh's (NINDS-LB) nerve conduction equation exhibits the qualitative features of axonal signaling. Even though it too is a nonlinear partial differential equation, its traveling wave solutions are obtained explicitly. This allows for complete parametric description of these solutions which correspond to a single nerve impulse and periodic trains of pulses. The results suggest that, for steady repetitive firing, the propagation speed of a periodic train depends on firing frequency. Additional numerical calculations are planned to further explore this dispersive aspect of nerve conduction.

As do other nerve conduction equations, the simple one has a multiplicity of traveling wave solutions. Such multiplicity contrasts with experimental observations and has led several investigators to conjecture on the stability of these solutions. For the simple equation, stability is analyzed explicitly to verify the conjectures and to indicate which waves should not be observed. Two different stability notions are formulated. Because they can lead to qualitatively different conclusions one should use the stability notion appropriate to the biophysical setting. Temporal stability is for perturbations imposed at a single instant of time. Spatial stability treats disturbances which may be continually imposed but spatially restricted to a fixed location. It is appropriate for many nerve signaling problems and is described in a paper to appear in the Biophys. J.

Stability results for the simple equation motivate their extension to a general class of nerve conduction equations. The class includes the Hodgkin-Huxley and FitzHugh-Nagumo equations. Critical stability transitions are analytically and parametrically characterized. For periodic wave trains, maximum frequency distinguishes the transition from spatial stability to instability. A manuscript has been submitted to the Journal of Mathematical Biology. The results were also presented by invitation at the Structural Stability Conference (Battelle Seattle Research Center) and will appear in the conference proceedings.

Keyword Descriptors:

Mathematical neural modeling, differential equations.

Publications:

Rinzel, J.: Voltage transients in neuronal dendritic trees. *Federation Proc.* *34*: 1350-1356, 1975.

Rinzel, J.: Spatial stability of traveling wave solutions of a nerve conduction equation. *Biophys. J.* (in press).

Serial No. Z01 AM 13,005-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Three dimensional reconstruction of macromolecules from electron micrographs

Previous Serial Number: NIAMDD, ODIR, MRB-6

Principal Investigator: Richard Gordon

Other Investigators: Marcello Barbieri (Guest Worker), Harvey B. Pollard and Lee Silver

Cooperating Units: RRB/NICHD; Department of Radiology, George Washington University School of Medicine

Man Years:

Total:	0.4
Professional:	0.4
Others:	0

Project Description:

The problem is to obtain three dimensional reconstructions of biologically important macromolecules from sets of tilted electron micrographs. The mathematical methods are those of reconstruction from projections.

Major Progress and Findings:

1) A set of interactive computer programs for three dimensional reconstruction and display of asymmetric biomolecules from electron micrographs was written and tested on chick ribosome dimers. These programs can be applied to any set of tilted micrographs.

2) Fortran subroutines were written for an inexpensive, accurate halftone display of images on graphics storage tube computer terminals.

3) A reconstruction algorithm was devised which uses all the available information on the spatial localization of every detected photon. This algorithm makes maximal use of gamma rays in 3D nuclear medicine. It is also applicable to single-count electron microscopy, and thus may contribute to solving the beam damage problem.

Significance to Biomedical Research:

Most enzymes have not been crystallized, and thus their structure cannot be determined by x-ray or neutron crystallography. The mathematics and computer programs being developed will permit us to reconstruct the 3D structure of individual, asymmetric macromolecules at moderate resolutions.

Proposed Course:

1) Three dimensional electron microscopy will be applied to dopamine-β-hydroxylase.

2) The single photon algorithm will be implemented and tested on a digital computer.

Keyword Descriptors: Reconstruction from projections, electron microscopy, ribosomes, macromolecules, image processing.

Publications:

Gordon, R. and Herman, G. T.: Three dimensional reconstruction from projections: a review of algorithms. In: Application of Optical Instrumentation in Medicine III, Society of Photo-Optical Instrumentation Engineers, 1975 2-14. (Reprint of an article in Int. Rev. Cytol. 38, 111-151, 1974.)

Serial No. Z01 AM 13,006-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Computer simulation of the change of shape of the newt neural plate during early embryogenesis

Previous Serial Number: MRB-6.1

Principal Investigator: Richard Gordon

Other Investigators: Antone G. Jacobson

Cooperating Unit: Zoology Department, University of Texas, Austin

Man Years:

Total:	0.2
Professional:	0.2
Others:	0

Project Description:

Objectives: The general aim of this project is to deduce the course of morphogenesis in vertebrate embryos from the behavior of their individual cells.

Progress: The first phase of the work was completed:

We have discovered the forces which are sufficient and necessary to produce the keyhole shape of the newt neural plate from a hemispheric sheet of cells one cell thick. We find that the joint operation of two forces effects this transformation: (1) a regionally programmed shrinkage of the surface of the neural plate (accomplished by contraction of the apical surfaces of the neural plate cells and elongation of the cells perpendicular to the plate); and (2) displacement of neural plate cells by elongation of the attached underlying notochord.

We have also (1) found that the lines of shear correlate with major developmental boundaries; (2) done a spatial and temporal analysis of unresolved tissue tensions in the ectoderm (which do not affect shaping of the neural plate); and (3) constructed a new fate map of the earliest neural plate.

Since each cell in the neural plate contributes to the motion of the whole sheet, we used a computer simulation and mathematical analysis ('morphodynamics') to deduce the morphogenesis of the neural plate from the behavior of its component cells.

The act of creating the computer simulation was not an end in itself, but rather sharpened our quantitative observation of and experiments on the embryo.

Significance to Biomedical Research: This work enables us to begin to make morphogenesis a deductive science. In addition to its broad application to problems in embryology, our study may be helpful in investigating the development of birth defects.

Proposed Course:

1) The mathods will be extended to a study of neurulation in the newt.

2) Computer programs will be obtained and developed to reconstruct embryos from serial sections and display the reconstructions in three dimensions.

Keyword Descriptors: Continuum mechanics, morphogenesis, vertebrate embryo, development, neural plate, morphodynamics.

Serial No. Z01 AM 13,007-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Development of striae in diatom shells

Previous Serial Number: None

Principal Investigator: Richard Gordon

Other Investigator: Ryan W. Drum

Cooperating Units: Fairhaven College, Bellingham, Washington

Man Years:

Total:	0.1
Professional:	.05
Others:	.05

Project Description:

Objectives: The ornate shells of diatoms form between two membranes. In the normal algae cell the pattern is reminiscent of those formed by a Turing reaction-diffusion mechanism. However, we have found aberrant colonies whose shells have multiply branching striae. These have given us a clue to the probable mechanism of striae growth.

Major Findings:

1) The pattern of the striae can be explained by two growth rules:

a) A stria which is too close to neighboring striae stops growing.

b) A stria with too much room branches. Otherwise striae grow at their tips at a constant rate.

2) A model has been formulated for the growth and branching of a stria based on instabilities in diffusion limited growth of an amorphous precipitate from a supersaturated solution.

Significance to Biomedical Research:

This study contributes to an understanding of one of the chemical bases for intracellular morphogenesis.

Proposed Course:

1) A quantitative fit of the growth rules to the observed striae patterns will be attempted.

2) A rigorous demonstration of the existence of instabilities in diffusion-limited growth will be sought, guided by the results of a Monte Carlo solution.

Keyword Descriptors: Morphogenesis, development, diatom shells, computer simulation.

Serial No. Z01 AM 13,008-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Feasibility study for a high resolution three dimensional x-ray breast scanner

Previous Serial Number: None

Principal Investigator: Richard Gordon

Cooperating Unit: Department of Radiology, George Washington University

Man Years:

Total:	0.1
Professional:	0.1
Others:	0

Project Description:

The problem is to find numerical methods which will allow three dimensional reconstruction of the breast so as to: 1) obtain 0.1 mm spatial resolution; 2) use relatively few mammograms taken at different angles; 3) be computationally feasible; 4) be implementable as an inexpensive x-ray breast scanner.

Major Findings: A method for dividing the space representing a computed cross-section has been found which is 10 to 20 times faster than existing methods and allows the finest details to be reconstructed:

"It is possible to reconstruct, in three dimensions, the finest structure in a set of two dimensional x-ray images or projections by resorting to two data reduction steps. Consider the reconstruction of a planar section. Each projection is then a one dimensional function. The first step is to approximate each projection as a sequence of step functions over intervals of varying width. This may be done by run length encoding and certain other bandwidth reduction schemes. Each interval corresponds to a ray across the reconstruction plane. Thus fine detail is represented by narrow rays, and broad features by wide rays. All the areas cut off by all the rays from all the projections constitute a partitioning of the reconstruction plane. The second reduction step is to represent each area (or equivalence class) in the plane by a single point.

Since the partitioning is unique for each set of projection data, it is necessary to have efficient algorithms to locate points to represent each area and to 'paint in' the final results on a display. The method generalizes easily to non-parallel rays."

For a fan beam in three dimensions the efficiency rises to as much as 125, based on a mean one dimensional run length encoding of 5.

Significance to Biomedical Research:

The study is aimed at mass screening for incipient breast carcinomas down to 0.1 mm diameter. An instrument based on these principles could significantly aid in early detection and diagnosis of breast cancer.

Proposed Course:

1) The above algorithm will be implemented on a computer and tested on a digitized x-ray contact print of a slice of human breast.

2) Algorithms will be developed for reconstructing small, high density carcinomas.

3) Two dimensional run length encoding schemes will be developed.

4) If the computational demonstration is successful, a breast scanner will be designed and built at George Washington University Hospital.

5) A body scanner based on the same principles will be considered.

Keyword Descriptors: Image processing, computerized tomography, breast cancer, x-ray scanner, reconstruction from projections.

Serial No. Z01 AM 13,009-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Monte Carlo methods for cooperative Ising models

Previous Serial Number: None

Principal Investigator: Richard Gordon

Man Years:

Total:	0.1
Professional:	.05
Others:	.05

Project Description:

A review article on the above subject has been prepared.

Abstract:

Monte Carlo computations are used to investigate the behavior of models which are too complicated for explicit mathematical evaluation. The Ising model falls in this category. Monte Carlo methods applicable to the Ising model deserve special study because of their wide applications to problems ranging from protein synthesis, membrane structure and transport, to the sorting out of embryonic cells.

The Ising model was originally designed to explain phase transitions of magnetic systems in terms of nearest neighbor interactions between spins arranged on a regular lattice. The spins were assigned one of two discrete values. Later the Ising model was applied to lattice gases, in which the discrete states of a lattice site were either 'empty' or 'occupied'. Since then the concept of an Ising model has been generalized through use to refer to any system with a finite or countable number of discrete, distinguishable states.

There is not just one Monte Carlo method but rather a diverse set of computational procedures which share in common the use of random numbers. The emphasis of this review will be on a careful separation and description of the Monte Carlo methods available for Ising systems.

Metropolis, Rosenbluth, Rosenbluth, Teller & Teller (1953) presented the first Monte Carlo method applicable to statistical mechanical systems at equilibrium. I shall present this classical method and its variations in some detail, as they apply to Ising systems. Certain restrictions of the classical method permit one to follow the dynamics of the approach to equilibrium.

The classical Monte Carlo method treats time as occurring in discrete steps. For Ising systems a more efficient computation is possible if time is treated as a continuous variable.

An exciting new prospect, stimulated by the availability of computer languages which can do algebra, is the budding development of Monte Carlo algebra. Methods falling in this class produce symbolic formulae instead of numbers, and are reviewed according to equilibrium and steady state algebraic methods applicable to Ising systems.

Another relatively unexplored Monte Carlo method matches the state of an Ising system to observable parameters.

The application of Monte Carlo methods for Ising models of mixed embryonic cells and polymer configurations are then discussed in detail.

Finally I discuss the minimization of errors and computational efficiency.

It is my intent to acquaint the reader with the full variety of Monte Carlo approaches to Ising models, and to stimulate interest in the search for new and more powerful methods.

Significance to Biomedical Research: This review provides a clear separation and description of the various approaches to Monte Carlo simulation of Ising systems. Such models are used to explain phenomena in membrane transport, membrane structure and excitability, protein synthesis, DNA unwinding, and cell sorting.

Keyword Descriptors: Ising models, numerical methods, Monte Carlo computing.

Publications:

Gordon, R., Goel, N. S., Steinberg, M. S. and Wiseman, L. L.: A rheological mechanism sufficient to explain the kinetics of cell sorting. In: Mathematical Models for Cell Rearrangement, editor G. D. Mostow, Yale University Press, New Haven, Chapter 11, pp. 196-230, 1975 (Updated reprint of an article in Journal Theoretical Biology 37, 43-73, 1972).

Serial No. Z01 AM 13,010-01 MRB
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Improved reconstructed images from the EMI Scanner

Previous Serial Number: None

Principal Investigator: Richard Gordon

Other Investigators: David O. Davis, David Rockoff, David Goodenough, Darrell Rigel and Lee Silver

Cooperating Unit: Department of Radiology, George Washington University

Man Years:

Total:	0.1
Professional:	0.1
Others:	0

Project Description:

The EMI-Scanner produces cross-sectional images of the brain from 180 to 225 scans with an x-ray beam. The reconstruction algorithm used, while very fast, does not make optimal use of the available data. The medical goal of this work is to maximize the use of the data. It is anticipated that images of equivalent diagnostic value will be obtained with less x-ray dosage than currently used.

Progress:

1) Computer programs have been written to transfer raw data and EMI reconstructions from the EMI to the NIH/DCRT PDP-10 computer.

2) Subroutines have been written to interface this data, efficiently stored on disc, to the SNARK reconstruction program.

3) A method has been devised to obtain finer resolution along the axis of rotation from detailed knowledge of the geometry of the A and B scans.

4) Richard Gordon was active on the steering committee of the NSF International Workshop on Three-Dimensional Image Reconstruction Techniques held at Brookhaven National Laboratory, 15-19 July, 1974. He has organized and will be chairing a Topical Meeting on Image Processing for 2-D and 3-D Reconstruction from Projections: Theory and Practice in Medicine and the Physical Sciences, to be held at Stanford University 4-7 August 1975 under the auspices of the Optical Society of America.

5) A comprehensive bibliography on reconstruction from projections has been nearly completed and will be published.

Significance to Biomedical Research:

The EMI-Scanner is in widespread use for the detection of tumors and other pathologies of the brain. This work can lead to a considerable reduction in the x-ray dosage and corresponding decrease in the time needed per patient, as well as to an improvement of the image quality.

Proposed Course:

1) A series of reconstructions will be made from decreasing amounts of raw EMI data. Their diagnostic value will be checked phychophysically.

2) An attempt will be made to detect and eliminate data gathered when the patient moves.

3) A computer program will be written for obtaining multiple planes from the A and B scans.

4) If successful, these programs may be incorporated into the computer running the EMI-Scanner at George Washington University Hospital.

Keyword Descriptors: Image processing, x-ray scanner, brain tumors, x-ray dose, computerized tomography, reconstruction from projections.

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMDD

July 1, 1974 to June 30, 1975

Phillip Gorden, M.D., Clinical Director

Clinical Investigations now consists of seven branches. The most recent being the Diabetes Branch formed from the Diabetes Section of the Clinical Endocrinology Branch. Dr. Jesse Roth is Chief of the Diabetes Branch and there are three additional senior investigators, Drs. Phillip Gorden, C. Ronald Kahn, and Matthew Rechler. The Digestive Diseases Branch has added a senior investigator to each of its sections. Dr. Anthony Jones has joined the Section on Liver Diseases and Dr. Denis McCarthy has joined the Section on Gastroenterology. This has led to an expansion of the clinical activities of the Digestive Diseases Branch. Dr. John Decker has been on sabbatical leave and Dr. Henry Metzger has been acting chief of the ARB. There have been no other major alterations of the senior staff.

We were all greatly saddened by the death of Dr. Norman Gordon from malignant lymphoma. Norman was one of our most outstanding Clinical Associates and he is greatly missed by all of his friends and colleagues in NIAMDD and throughout NIH.

Both nursing units 8-West and 9-West were closed for five weeks each for the purpose of installing new electrical, oxygen and suction equipment. Even with the loss of these units and the necessity to limit admissions, there was an overall increase in admissions over the previous year, reflecting primarily an increase in admissions to 8-West which accommodates the Diabetes Branch, Clinical Endocrinology Branch, and the Metabolic Diseases Branch.

For the period April 1, 1974 to March 31, 1975, the statistics with regard to patient admissions and outpatient visits are as follows: There were 575 inpatient admissions for a total of 13,995 hospital days, yielding an average length of stay of 26 days. The average bed occupancy was 60%. There were altogether 3,376 outpatient visits to the several clinics operated by NIAMDD.

Data on the inpatient admissions have been sorted by computer and give rise to the following tables, which may be informative:

	Admissions by Nursing Unit
9-East	220
9-West	135
8-West	220
Total	575

Admissions by Branch		Assigned Beds	Admissions per Bed	Clinical Associates on Duty*
CEB DB MDB	217	20	10.9	6
ARB	193	15	12.9	7
DDB	97	14	6.0	7
PMB	61	6	10.1	1
CHB	7	7	1.0	2
Total	575	62	9.3	23

Admissions by Admitting Diagnosis	
Acromegaly	17
Cystic fibrosis	47
Diabetes	26
Gilbert's syndrome	7
Growth retardation	9
Hepatitis	11
Hereditary spherocytosis	8
Hypopituitarism	8
Normal volunteers	54
Rheumatoid arthritis	41
Systemic lupus erythematosus	107
Thyroid carcinoma	20
Thyroid, other disorders	7
Wilson's disease	13
Zollinger-Ellison syndrome	5
Others	195
Total	575

During FY 1975 there were 23 Clinical Associates on duty with NIAMDD. Other temporary professional and nonprofessional staff included 8 Visiting Scientists and 31 guest workers, representing 12 foreign countries in addition to the United States.

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*Does not include Clinical Associates rotating from other Institutes, nor guest workers.

ARTHRITIS AND RHEUMATISM BRANCH

Investigations in ARB cover the gamut of direct therapeutic studies on human patients, use of animal models to discover pathogenetic mechanisms and fundamental studies designed to develop new investigative tools and increase our knowledge about normal biochemical and immunologic mechanisms.

I. Therapeutic Trials

A. No satisfactory therapy exists for severe manifestations of Systemic Lupus Erythematosus (SLE) except for renal disease and even the latter does not respond to conventional therapy when the creatinine clearance is markedly reduced. A controlled trial of intravenous cyclophosphamide in patients with severe renal disease, and/or extra-renal manifestations of SLE has been initiated (Plotz, Decker, Steinberg) as part of a continuing search for effective therapy of this serious disease. Patients with less malignant forms of the disease were found (Steinberg, Decker) to respond more favorably to immunosuppressive agents (cyclophosphamide, azathioprine) than do conventional (anti-inflammatory drugs) regimens. Long term followup of the patients in the latter studies is underway and further patients will be admitted to a carefully randomized study of alternative treatment schedules.

B. Patients with Psoriasis may develop severe arthropathy. A randomized double-blind controlled trial with azathioprine and methotrexate has been initiated (Plotz, Steinberg, Milstone (CC:DIR), Black (CC:DIR), Gerber, Lucky Paget).

II. Disease Mechanisms - Human

A. Continuing studies on an SLE-like disease in mice (see below) have demonstrated a variety of immunologic abnormalities. Based on these findings a study has been initiated (Steinberg, Gershwin, Morton, Glinski (Dermatology Branch, NCI), Brady (Indian Health Service)) in which patients with SLE, their families and populations with a high incidence of SLE will be examined for corresponding findings. It has been found that 1) Particular groups of American Indians (Sioux, Crow, Arapahs) have an increased annual incidence of SLE and these will be intensively studied. 2) Patients with active SLE have a relative decrease in a subpopulation of T-cells and an increase in "null" cells (cells which are missing characteristic markers of both T and B cells). Such "active" patients also have anti-lymphocyte antibodies which can reduce the number of peripheral T cells and interfere with their function. In other studies it was found that the avidity of anti-nucleic acid antibodies correlated with the presence of active renal disease and therapy led to a concordant decrease in renal manifestations and precipitating antibodies (Steinberg, Gershwin, Gerber, Scher (NNMC), Lindsley (Walter Reed), Hyman (Walter Reed)).

B. The synovial membranes of patients with Rheumatoid Arthritis (RA) are infiltrated with lymphocytes which may be intimately involved in the autodestructive joint lesions. These lymphocytes turn out to be predominantly

(70%) T-cells - i.e., the same as the lymphocytes in normal blood (van Boxel, Paget). The cytotoxic capacity of these intra-lesional lymphocytes is being assessed.

"Rheumatoid factor" (anti-IgG antibodies) in RA patients is a pathognomonic finding. The specificity of these antibodies has remained uncertain (i.e., are they directed to normal IgG or to altered IgG). The recent findings (Eisenberg, Plotz) that the preferential binding of these antibodies to aggregated IgG can be simply accounted for by multi-point attachment strongly suggests that an "altered" IgG as the putative antigen must not be postulated.

C. The mucopolysaccharidoses and mucolipidoses are a group of genetically determined disorders of lysosome function, which have been under intensive investigation by Dr. Neufeld's group. Two forms of iduronidase (one of the defective enzymes in such disorders) have recently been characterized and studies of them have confirmed the previous suggestion that uptake by cells of these enzymes requires particular structural features of these proteins. Another enzyme iduronate sulfatase can now be assayed for simply (Lim, Leder, Bach, Neufeld) using serum. This obviates the need to culture fibroblasts in order to diagnose the Hunter syndrome and differentiate patients with this disorder from mucolipidosis II and III. Using lymphocytes, Hunter and Hurler patients can be distinguished. These findings are of considerable usefulness for genetic counselling.

D. Chronic Lymphatic Leukemia (CLL) is thought to be a malignancy of bone-marrow derived (B) lymphocytes. Recently a patient was examined who had a circulating IgG paraprotein associated with the leukemic process. The protein-specific (idiotypic) antigenic determinants were found on more than 90% of this patient's cells (Schroer (Wash. Univ.), Briles (Wash. Univ.), van Boxel, Davie (Wash. Univ.)). This represents some of the most convincing data that CLL represents a clonal lymphoproliferation.

E. The surface membranes of lymphocytes of some patients with Sjögren's syndrome have multiple heavy-chain types of immunoglobulins (van Boxel, Hardin (NIDR), Green (NIAID), Paul (NIAID)). It has now been found that these probably represent anti-lymphocyte antibodies (Paget, Chused (NIDR), van Boxel). Possibly these are related to the pathogenesis of this disease. The significance of these antibodies is under study.

III. Disease Mechanisms - Animal Models

A. New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. This animal model has been pursued in this Branch for 10 years. Recently the role of a subpopulation of T-cells, suppressor cells - has been actively explored. Abnormalities in this subpopulation were found (Gerber, Hardin (NIDR), Chused (NIDR),Steinberg. Other studies showed 1) that spleen cells from normal and affected mice show a similar fractionation pattern by sedimentation analysis but functional differences are observable. 2) T-cell deficient "nude" mice develop an autoimmune syndrome involving anti-DNA antibodies and immune complex glomerulonephritis (Morse (NIAID), Steinberg, Schur (not NIH), Reed (not NIH)). Nude mice bred on a background with virus-infected Balb/c mice show autoimmunity while the nude gene on a non-virus carrying Swiss genetic background did not. Such studies may help to disentangle the relationship between the viral and genetic components of certain autoimmune disorders. 3) Therapy with conconavalin A has prolonged tolerance to proteins in NZB/W mice and corrected a number of immunologic abnormalities of older New Zealand mice (as did non-specific adjuvants and thymosin) (Gershwin, Ahmed (not NIH), Steinberg, Thurman (not NIH), Goldstein (not NIH)). A variety of more conventional therapeutic regimens are also being tested (Steinberg, Gerber, Gershwin, Gelfand (NIAID)) to serve as models for human studies.

B. Autoimmune thyroiditis is a classic model for human autoimmune disease. The lymphocytic infiltrates in guinea pigs with induced disease were found to have similar proportions of T- and B- cells as found in the peripheral blood (Paget, van Boxel). The cytotoxic capacity of these cells for thyroglobulin coated target cells is being assessed.

IV. Fundamental Studies

A. "Activated" T-cells were recently shown to have surface receptors for IgG Fc regions (van Boxel, Rosenstreich (NIDR) similar to those observed on B-cells. Studies to determine if this is principally due to a T-cell subpopulation (helper, suppressor, cytotoxic) are underway.

B. IgD is a commonly found surface Ig on lymphocytes. Its function is unknown. Recently it was observed that treatment of cells with anti-IgD 1) did not stimulate transformation, 2) did not affect the mixed lymphocyte cytotoxic reaction but, 3) had an inhibitory effect on Ig production by lymphocytes (van Boxel, Blaese (NCI), Broder (NCI), Waldmann (NCI)). This raises the interesting possibility that IgD serves a regulatory role in lymphocyte physiology.

C. Bilirubin and other substances such as thyroxine and salicylates bind tightly to plasma proteins and cannot be removed by dialysis. Affinity chromatography methods using albumin conjugated agarose beads are being explored as a useful procedure (Plotz, Scharschmidt, Berk). In vivo studies on rats, monkeys and sheep have been begun in anticipation of human applications.

D. Aggregates of immunoglobulins are implicated in a variety of immunological phenomenon. It would be extremely useful for the study of such events to have available stable well-defined antigen-antibody complexes. The usefulness of a bivalent affinity-labeling reagent in this regard is being explored (Plotz).

E. The mechanism by which antigen-induced antibody-mediated reactions lead to complement fixation, lymphocyte transformation and mast cell degranulation is unknown. Few systems exist in which these phenomena can be rigorously studied ultimately at the molecular level. A promising system has now been developed by which the interaction of IgE with basophil/mast cell surface receptor studied (Metzger, Isersky, Kulczycki, Carson, Halper, Budman, Mendoza, Siraganian (NIDR), Buell (NCI). The mechanism, kinetics, and specificity of IgE binding have been defined; the number of receptors during various phases of cell growth determined and active cell free membrane particles isolated. Attempts to isolate and purify the receptor and to find functional correlates of cell activation are under way.

Project No. Z01 AM 41000-06 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

Previous Serial Number: NIAMDD-ARB-19c

Principal Investigators: Dr. John L. Decker and Dr. Alfred D. Steinberg

Other Investigators: Dr. Paul H. Plotz, Dr. Jack Klippel

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

Two previous ten week studies by our branch suggested that cyclophosphamide was effective in the treatment of lupus nephritis and that azathioprine was less effective. A long-term study of these patients is being carried out to determine whether or not one therapeutic program is superior over the course of several years. In addition, newer drug programs have been suggested by study in New Zealand mice.

Methods Employed:

Patients meeting predefined criteria for diagnosis of SLE and activity of nephritis will be admitted to the trial. In a baseline control period, the patients will be kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These will be continued into the study period with as little change as possible and with the addition of randomly assigned 1) cyclophosphamide, 2) azathioprine, 3) cyclophosphamide plus azathioprine, 4) intravenous cyclophosphamide, 5) steroids only.

A large number of serological, chemical and urine values will be serially obtained and the patients will be followed for life. Immunoglobulin levels, anti-DNA antibodies, and serum complement are among those measures. We will determine time to death or renal dialysis in each treatment program and assess the toxicity as well as efficacy of each program.

Major Findings:

Patients receiving no immunosuppressive drug have progressed to death or dialysis at a rate faster that those recieiving drug. Significant differences have not yet been reached between any drug programs. Toxicity from cyclophosphamide appears to be similar in frequency and severity to that from azathioprine.

Significance to Bio-Medical Research and the Program of the Institute:

If difference in the response of the disease and some of the immunological processes can be detected between different drug programs, it will be possible to draw conclusions with reference to the cell populations involved in systemic lupus erythematosus and thus increase our understanding of the disease process itself. It is expected that the results will permit us to select among treatment programs in clinical management.

Proposed Course:

A long-term followup of these patients will be continued, primarily by one-week admissions every 6 months. New patients will continue to be admitted to the 5 study groups.

Keyword Descriptors: lupus nephritis,
immunosuppressives,
autoimmunity

Honors and Awards: None

Publications:

1. Steinberg AD, Decker JL: A double blind controlled trial comparing cyclophosphamide, azathioprine and placebo in the treatment of lupus glomerulonephritis. Arthritis Rheum., 17:923, 1974.

2. Decker JL, Steinberg AD, Gershwin ME, Seaman WE, Klippel JH, Plotz PH, and Paget SA: Systemic lupus erythematosus: Contrasts and Comparisons. Ann. Intern. Med. 82:391, 1975.

Project No. Z01 AM 41001-04 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo

Previous Serial Number: NIAMDD-ARB-18c

Principal Investigator: Paul H. Plotz, M.D.

Other Investigators: John L. Decker, M.D. and Alfred D. Steinberg, M.D.

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

At present no satisfactory therapy exists for severe manifestation of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high dose of prednisone (up to 2 mg per kg for 3 weeks).

Methods Employed:

Patients with 1) severe renal disease or rapid deterioration of renal function, 2) sensitivity to azathioprine precluding the CAP protocol, 3) severe extra-renal lupus (seizures, hemorrhage, psychosis, hemolytic anemia, myocarditis, or pericarditis uncontrolled by high-dose prednisone) will be randomly assigned to receive intravenous cyclophosphamide or placebo for 3 initial injections over 6 weeks and then at 3 month intervals. Renal and non-renal patients will be separately randomized. The study will be double-blind: neither physicians nor patients will know which therapy is being administered. The end point of the study will be death or the need for renal dialysis except that patients well for 1 year may have therapy stopped. The potential hazards and benefits will be explained in full to all patients or their families.

Project No. Z01 AM 41001-04 ARB

Major Findings:

Six patients have been admitted to the study so far - two in the non-renal groups and four in the renal group. No findings of significance to date.

Significance to Bio-Medical Research and the Program of the Institute:

The therapy of SLE has been under study here for several years. The present study is an attempt to extend the therapy for patients unresponsive to conventional therapy.

Proposed Course:

We plan to enter 20 to 30 patients into the study over the next 3-5 years.

Keyword Descriptors:

Systemic lupus erythematosus, connective tissue disease, collagen-vascular disease, cytotoxic drug, cyclophosphamide, nephritis.

Honors and Awards: None

Publications: None

Project No. Z01 AM 41002-10 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Retrospective Evaluation of Knee Synovectomy in Rheumatoid Arthritis

Previous Serial Number: NIAMDD-ARB-2c

Principal Investigator: John L. Decker, M.D.

Other Investigators: Robert G. Aptekar, M.D., Leonard T. Peterson, M.D. and David Fried, M.D.

Cooperating Units: George Washington University School of Medicine, Washington, D. C. and Clinical Center Rehabilitation Department.

Project Description:

This project has been delayed due to the sabbatical of the Principal Investigator.

Project No. Z01 AM 41003-02 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Synovial Changes in Rheumatoid Arthritis Under Cyclophosphamide Therapy

Previous Serial Number: NIAMDD-ARB-1c

Principal Investigator: John L. Decker, M.D.

Other Investigators: Robert G. Aptekar, M.D., Hereward Cattell, M.D., and Hope Hopps.

Cooperating Units: George Washington University Hospital and Bureau of Biologics (DIR)

Project Description:

This project has been delayed due to the sabbatical of the Principal Investigator.

Project No. Z01 AM 41004-10 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Role of Infection in Rheumatoid Disease

Previous Serial Number: NIAMDD-ARB-3c

Principal Investigator: John L. Decker, M.D.

Other Investigators, Robert G. Aptekar, M.D.

Cooperating Units: None

Project Description:

This project has been completed. A fourth manuscript has been accepted for publication and work is being done on a fifth manuscript.

Decker JL and Barden JA: Mycoplasma hyorhinis Arthritis of Swine: A Model for Rheumatoid Arthritis. Rheumatology (in press).

Project No. Z01 AM 41005-03 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1974

Project Title: Cellular and humoral immunity in systemic lupus erythematosus (SLE) undergoing randomized cytotoxic therapy.

Previous Serial Number: NIAMDD-ARB-5c

Principal Investigator: John L. Decker, M.D.

Other Investigators: Alfred D. Steinberg, M.D., Robert G. Aptekar, M.D., and Thomas A. Waldmann, M.D. (C:MET)

Cooperating Units: National Cancer Institute, Metabolism Branch.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This study has been completed and a manuscript is in preparation.

Project No. Z01 AM 41006-02 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization of infiltrating lymphocytes in rheumatoid synovial membranes

Previous Serial Number: NIAMDD-ARB-11C

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : Stephen Paget, M.D., John L. Decker

Cooperating Units :

Man Years:

Total :	3/4
Professional:	1/2
Other :	1/4

Project Description:

Objectives:

Immunological mechanisms in the pathogenesis of rheumatoid arthritis may be revealed by study of the remarkably dense lymphocytic infiltrations in rheumatoid synovial membranes. We aim to determine the B and T cell composition of this infiltrate and will attempt to assess some of the functional capacities of these intralesional lymphocytes. A major question is whether the lymphocytes are engaged in an autodestructive process.

Methods Employed:

As previously described we developed a method for the extraction of viable lymphocytes by digestion of synovium with collagenase and deoxyribonuclease. B and T cells were quantitated in these populations using standard procedures. In addition adherence of C sensitized erythrocytes to lymphocytes with C3 receptors (B cells) in cryostat tissue sections were studied to determine the tissue distribution of B cells.

Major Findings:

A wide spectrum from fibrosis to dense lymphocytic infiltrates were found in the sixteen synovia examined. T cells were found to predominate (70%) while most of the remainder of the lymphocytes consisted of B cells (10-35%) with a varying proportion of cells with neither marker (null cells). Study of tissue sections corroborated these findings in that the majority of lymphocytes did not show adherence of C sensitized red cells. However, occasional typical follicular-type accumulations of lymphocytes showed dense red cell adherence supporting previous suggestions based on histological criteria, that true lymphoid follicles with germinal centers occur in rheumatoid synovial infiltrates. Extraction of lymphocytes with collagenase and purification using velocity sedimentation may have general applicability as methods for the study of intralesional lymphocytes.

Significance to Bio-Medical Research and the Program of the Institute:

There is much evidence for immunological mechanisms having a role in the pathogenesis of rheumatoid arthritis. Since B and T cells form functionally as well as ontogenetically distinct populations, study of intralesional B and T cells may provide information concerning pathogenetic mechanisms. Striking clincial improvement in rheumatoid arthritis has been noted following thoracic duct drainage, a procedure depleting primarily T cells; is not inconceivable that this manoevre is effective because it depletes synovial T cells, which have been shown to have the capacity to destroy target cells *in vitro*.

Proposed Course:

Attempts will be made to assess the cytotoxic capacities of these isolated intralesional lymphocytes in tissue culture.

Keyword Descriptors:

Rheumatoid synovial membranes, intralesional lymphocytes, surface markers.

Honors and Awards: None

Publications:

1. van Boxel, J. A. and S. A. Paget: Extraction, purification and characterization of surface markers of lymphocytes infiltrating rheumatoid synovial membranes. J. of Rheum 1:87, 1974.

2. van Boxel, J. A. and S. A. Paget: Predominantly T-cell infiltrate in rheumatoid synovial membranes. Submitted for Publication.

Project No. Z01 AM 41007-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 though June 30, 1975

Project Title: Cell surface Immunoglobulins of lymphocytes in Sjögrens Syndrome

Previous Serial Number: None

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : Stephen Paget, M.D.

Cooperating Units : None

Man Years:

Total :	3/4
Professional:	1/2
Other :	1/4

Project Description:

Objectives:

In a previous report we presented evidence for multiple immunoglobulin (Ig) classes (IgM, IgG, IgA and IgD) on the surface membranes of individual lymphocytes in some patients with Sjögrens Syndrome. The mechanisms responsible for this phenomenon are not clear, and have been investigated.

Methods Employed:

Class-specific fluorescein and rhodamine tagged antibodies were used to demonstrate surface Ig (B cells) and T-cells were identified by formation of rosettes with sheep erythrocytes. Procedures used for double labelling and co-capping experiments, as described previously.

Major Findings:

We have found that incubation of lymphocytes from normal controls with serum from patients whose lymphocytes bore multiple H-chains resulted in an increased proportion of the normal lymphocytes staining with some class-specific antisera, suggesting that anti-lymphocyte antibodies might account for the multiple Ig classes observed. While this was demonstrated for IgG and IgM, the number of cells staining with anti-IgA and anti-IgD did not increase despite collection of blood in the presence of e-amino captoic acid to prevent possible breakdown of the IgD. When normal lymphocytes were incubated with sera from such patients after prior capping with fluorescein conjugated anti-kappa chain antibody, 50% of the cells stained diffusely with anti-IgM antisera, suggesting that anti-lymphocyte activity was directed at a non-Ig B-cell membrane component. However the percent of cells that stained exceeded the total B cell frequency suggesting that activity was also directed at a subpopulation of T-cells, and this was confirmed when 5-10% of stained cells formed spontaneous E-rosettes with sheep erythrocytes (a T cell marker). Normal lymphocytes could also be stained directly with the fluorescein conjugated IgG fraction of serum from one such patient. Although reagents were ultracentrifuged, a significant proportion of cells staining due to binding antigen--antibody complexes to Fc receptors on the lymphocytes is not ruled out. Serum from such patients was not found to have a specific inhibitory effect on the mixed lymphocyte reaction.

Significance to Bio-Medical Research and the Program of the Institute:

These findings suggest the presence of antibodies in some patients with Sjögren's syndrome which are directed to a subpopulation of B and T lymphocytes and which might account in part for multiple H-chains found on their lymphocytes. The nature of the membrane antigen and the subpopulation of lymphocytes bearing this proposed antigen are of interest, and may have implications for certain aspects of the pathogenesis of the disease.

Proposed Course:

Studies will be undertaken using the $(\mathrm{Fab}^1)_2$ fragment of the IgG fraction of this patient's serum, which will be tested for activity so as to provide more definitive evidence for antibody activity.

Keyword Descriptors:

Sjögrens syndrome, lymphocyte, cell surface immunoglobulin

Honors and Awards: None

Publications:

1. van Boxel, J. A., J. A. Hardin, I. Green and W. E. Paul: Multiple heavy chains on individual B-lymphocytes in the peripheral blood of patients with Sjögrens Syndrome. New Engl. J. Med. 289: 823-827, 1973.

2. Paget, S. A., T. M. Chused, and J. A. van Boxel: Evidence for anti-lymphocyte antibodies in Sjögren's Syndrome. Abstr ARA meetings 1975.

Project No. Z01 AM 41008-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Characterization of infiltrating lymphocytes in experimental autoimmune thyroiditis of the guineapig

Previous Serial Number: None

Principal Investigator: John van Boxel, M.D.

Other Investigators : Stephen Paget, M.D.

Cooperating Units : None

Man Years:

Total :	3/4
Professional:	1/2
Other :	1/4

Project Description :

Objectives:

The B & T cell composition of the lymphoid infiltrate in cell-mediated immunological reactions is unknown. We have investigated the infiltrate in experimental autoimmune thyroiditis in the guineapig, a lesion generally considered to be a typical cell-mediated reaction, in that it is transferable to normal recipients by cells but not by serum. The techniques developed allow for the extraction of the infiltrating cells in a viable state, so that functional aspects of the intralesional lymphocytes can be determined; although there is now abundant evidence for the tissue damaging capacity of both T and non-T lymphocytes *in vitro*, these findings have yet to be convincingly shown to have relevance *in vivo*. Furthermore, characterization of the intralesiona lymphocytes in this easily studied animal model may provide insight into the nature of the lymphocyte infiltrate in rheumatoid synovial membranes (see above) in which the nature of the inflammatory infiltrate, while also chronic and predominantly lymphocytic, is unknown.

Methods Employed:

Thyroiditis was induced by injection of thyroid extract in complete Freund adjuvant. Single or pooled glands were minced finely and digested with collagenase and deoxyribonuclease, and the lymphocytes in the resulting single cell suspension examined for B and T-cell markers, and compared with peripheral blood and lymph-node lymphocytes. T-cells were identified by spontaneou rosette formation with rabbit erythrocytes and B-cells were detected by immunofluorescence staining for easily detectable surface immunoglobulin.

Major Findings:

The predominant lymphocyte was found to be the E-rosette forming T-lymphocyte, accounting for over 70% of the cells, while most of the remainder were Ig-bearing B-cells. A varying percentage of cells lacked both markers and may represent T-cells. These proportions were similar to that found in peripheral blood and indeed also in lymph-node; although this may be coincidental, it could conceivably indicate random emigration of lymphocytes into the lesion. Preliminary data from studies in tissue culture indicate that there are cells in the thyroid infiltrate with the capacity to mediate antibody-dependent cytotoxicity.

Significance to Bio-Medical Research and the Program of the Institute:

Very little is known about the B and T cell composition of chronic inflammatory lesions, in particular cell-mediated immune reactions. Although the presence of B cells and T-cells have been noted in other studies in some lesions, no previous study has quantitated the relative proportions of these cells. This model for the study of lymphocytes in a cell mediated reaction allows for the study of viable cells in tissue culture which may lead to increased understanding of the mechanisms involved.

Proposed Course:

Attempts will be made to assess the capacity of both infiltrating T and non-T cells to lyse thyroglobulin-coated target cells.

Keyword Descriptors:

Experimental autoimmune thyroiditis, guineapig, infiltrating lymphocytes, surface markers.

Honors and Awards: None

Publications:

1. Paget, S. A. and van Boxel, J. A.: Experimental autoimmune thyroiditis in the guineapig characterization of intralesional lymphocyte populations. Fed. Proc. 34:950, 1975.

Project No. Z01 AM 41009-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of IgD on cord blood lymphocytes and mouse lymphocytes

Previous Serial Number: None

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : Fred D. Finkelman, M.D. (I:LI), Richard Asofsky, M.D. (I:LMI) and William E. Paul, M.D. (I:LI)

Cooperating Units : Laboratory of Immunology and Laboratory of Microbial Immunity, NIAID

Man Years:

Total :	1/2
Professional:	1/4
Other :	1/4

Project Description:

Objectives:

IgD appears to be the major surface immunoglobulin on neonatal (cord blood) lymphocytes. This fact may point to a biological role for this molecule; in addition the high proportion of IgD bearing lymphocytes facilitates study of these cells. Efforts to establish a function for cell-membrane IgD have been hampered by the lack of animal models, as IgD has only been identified in man.

Methods Employed:

Cell membrane Ig was analyzed by 1) cocapping studies using antibodies coupled to different fluorochromes (rhodamine and fluorescein). 2) Surface iodination techniques in which Ig is extracted with a non-ionic detergent, precipitated with anti-Ig serum and electrophoresed on SDS polyacrylamide gels.

Major Findings:

1. A high frequency of IgD bearing cells in some specimens of cord blood appear to bear IgG in addition to IgM.
2. Recently an Ig class on mouse lymphocytes has been found by surface iodination techniques which has been proposed as a homologue of IgD.
   a) Cocapping experiments appear to provide confirmatory evidence for

this: following capping with anti-μ, anti-kappa antiserum produced diffuse staining of a large proportion of mouse lymphocytes which was not accounted for by IgA or IgG.

b) Iodination and extraction procedures followed by gel electrophoresis confirmed the presence of an H chain with mobility more rapid than that of μ chain (putative IgD), although this was not found in rabbit or guinea pig. Delta chain identified on cord blood lymphocytes had a mobility similar to human and mouse μ chain, however.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of IgG on IgD bearing cord blood lymphocytes if confirmed is contrary to the current concept of IgD sharing the cell membrane exclusively with IgM. Evidence is presented that appears to support the presence of an IgD homologue in the mouse, using immunofluorescence and surface iodination procedures.

Proposed Course:

Efforts will be made to raise an antiserum to mouse "IgD" to facilitate the study of its biological function.

Keyword Descriptors:

Immunoglobulin-IgD, cell surface immunoglobulin, cord blood, mouse.

Honors and Awards: None

Publications: None

Project No. Z01 AM 41010-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effect of purified antibody to IgD on lymphocyte transformation and immunoglobulin production.

Previous Serial Number: None

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : Michael R. Blaese, M.D. (C:MET), Samuel Broder, M.D. (C:MET) and Thomas A. Waldman, M.D. (C:MET)

Cooperating Units : Metabolism Branch, NCI

Man Years:

Total :	1/2
Professional:	1/4
Other :	1/4

Project Description:

Objectives:

We were previously the first to demonstrate that IgD, an immunoglobulin class of as yet unknown function, is bound to the surface membrane of an unexpectedly high frequency of human lymphocytes. So as to investigate the biological role of IgD the effects on purified antibody to IgD was tested in various *in vitro* systems for possible stimulator or inhibitor effects.

Methods Employed:

IgD myeloma protein was purified using DEAE cellulose chromatography followed by G 200 gel filtration. Goat antibody to IgD was purified by affinity chromatography. Anti-IgD was added to cultures of human lymphocytes and to the mixed lymphocyte reaction and effects monitored by standard methods of isotope incorporation. Ig secretion was measured by radioimmunoassay.

Major Findings:

Contrary to previous reports we found that highly purified anti-IgD did not stimulate cells to transform. No effect was noted on the MLC reaction. Preliminary data suggest an inhibitory effect on Ig production.

Significance to Bio-Medical Research and the Program of the Institute:

IgD is now considered to be a major cell membrane immunoglobulin, yet there is no clue as to its function. It exists on cells largely together with IgM and most likely functions as a receptor. The finding of interference by anti-IgD in these systems, if confirmed, may imply a regulator role for the proposed IgD receptor.

Proposed Course:

Investigations will be directed at elucidating the significance of the *in vitro* effect of anti-IgD.

Keyword Descriptors:

Anti-IgD antibody, lymphocyte transofrmation, Ig production.

Honors and Awards: None

Publications: None

Project No. Z01 AM 41011-02 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Binding of aggregated γ-globulin to T-lymphocytes

Previous Serial Number: NIAMDD-ARB-12C

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : David L. Rosenstreich, M.D.

Cooperating Units : Laboratory of Microbiology and Immunology/NIDR

Man Years:

Total :	1/2
Professional:	1/4
Other :	1/4

Project Description:

Objectives:

We have previously shown in the guineapig that a subpopulation of thymus-derived or T-lymphocytes have the capacity to bind aggregated γ-globulin, a property hitherto believed to be exclusive to bone-marrow derived or B-cells. The findings suggested that T cells developed an Fc receptor upon activation. We aim to extend these findings to determine (1) Whether T cells (in man) develop an Fc receptor upon allogeneic stimulation *in vitro* (2) Whether activated T-cells with Fc receptors can mediate antibody - dependent cytotoxicity by virture of their Fc receptor.

Methods Employed:

1. Mixed lymphocyte reactions using human peripheral blood lymphocytes were set up using standard procedures. Aggregated guineapig γ-globulin was used to detect the putative Fc receptor and simultaneous sheep cell rosettes used as a marker for T cells.
2. Peritoneal exudate cells were allowed to form T cell rosettes, purified by gradient sedimentation and tested for antibody dependent cytotoxicity using previously described methods.

Major Findings:

1. Allogeneically stimulated human T cells develop the capacity to binding aggregated γ-globulin *in vitro* and hence presumably acquire Fc receptors.

2. Despite the apparent presence of Fc receptors, activated T cells do not appear to have the capacity to mediate antibody-dependent cytotoxicity.

Significance to Bio-Medical Research and the Program of the Institute:

Fc receptors on T cells may well play a major role in basic immunological reactions such as for example antigen processing. Furthermore, reports of the existance of T cell immunoglobulin may be explained by adherence of exogenous Ig via this receptor. The finding of T cells with apparent Fc receptors in the MLC reaction extends these findings to an in vitro model in man. Lack of cytotoxicity by activated T cells will further narrow down the search for the killer (K) cell in antibody-dependent cell-mediated cytotoxic system.

Proposed Course:

Using the model systems described we will attempt to provide answers to the questions (1) Do Fc receptor bearing T cells fall into one of the recently described functional categories of T cells--helper, suppressor, cytotoxic (2) Do anti Ia antisera block T cell Fc receptors as has been shown for B cells.

Keyword Descriptors:

T lymphocytes, Fc receptor, guinea pig, aggregated immunoglobulin.

Honors and Awards: None

Publications:

1. van Boxel, J. A., and D. L. Rosenstreich: Binding of aggregated γ-globulin to activated T lymphocytes in the guinea pig. J. Exp. Med. 139-1002, 1974.

Project No. Z01 AM 41012-02 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antibody-dependent lymphoid cell mediated cytotoxicity

Previous Serial Number: NIAMDD-ARB-14C

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : None

Cooperating Units : None

Project Description : This study has been incorporated in Project # Z01 AM 41011-02 ARB.

Project No. Z01 AM 41013-02 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cell Surface Immunoglobulins

Previous Serial Number: NIAMDD-ARB-13C

Principal Investigator: John A. van Boxel, M.D.

Other Investigators : Donald Buell, M.D.

Cooperating Units : Immunology Branch/NCI

Project Description : This project has been completed and led to a third publication.

1. van Boxel, J. A. and D. Buell: IgD on cell membranes of human lymphoid cell lines with multiple immunoglobulin classes. Nature 251: 443-444, 1974.

Project No. Z01 AM 41014-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cell surface immunoglobulins in chronic lymphocytic leukemia

Previous Serial Number: None

Principal Investigator: John van Boxel, M.D.

Other Investigators: Kenneth R. Schroer, M.D., David E. Briles, M.D., and Joseph M. Davie, M.D.

Cooperating Units: Departments of Pathology and Microbiology,
Washington University School of Medicine,
St. Louis, MO.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives:

Chronic lymphatic leukemia is thought to be a malignancy of bone-marrow derived (B) lymphocytes, since the leukemic cells from a majority of patients bear easily detectable membrane-bound immunoglobulin (Ig). In most but not all studies, cells from an individual patient bear Ig restricted to a single light chain class so that it is suspected that the CLL cells represent the progency of a single class of B-cells. The aim of the present study was to determine whether the Ig on CLL cells possess the same idiotypic (variable region) determinants to provide further evidence on this point.

Methods Employed:

Anti-idiotypic serum was prepared against the purified circulating IgG paraprotein of a single patient with CLL by passage of antiserum over an immunoabsorbent column of human γ-globulin coupled to sepharose 4B. Specificity was established by hemagglutination inhibition using normal Ig, IgG myeloma proteins, as well as Fab fragments and H and L-chains and recombined H and L-chains of the paraprotein. Anti-idiotypic serum was fluorescein-tagged and the cells of the patient and controls stained to detect membrane-bound idiotypic determinants.

Major Findings:

Virtually all the CLL cells were shown to possess the same antigenic determinants that the paraprotein possessed including the idiotype. That is, anti-idiotype serum stained >90% of this patient's cells, but not the cells of various normal donors and other patients with CLL.

Significance to Bio-Medical Research and the Program of the Institute:

This study was the first to show idiotypic informity of cell-surface immunoglobulin in CLL, and this may represent the best evidence to date to support the thesis that the lymphoproliferation in CLL is monoclonal.

Proposed Course:

This project has been completed and led to a publication.

Keyword Descriptors:

Chronic lymphocytic leukemia, idiotypic uniformity, cell surface immunoglobulin.

Honors and Awards: None

Publications:

Schroer KR, Briles DE, van Boxel JA, Davie JM: Idiotypic uniformity of cell surface immunoglobulin in chronic lymphocytic leukemia: Evidence for monoclonal proliferation. J Exp Med 140:1416-1420, 1974.

Project No. Z01 AM 41015-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Therapeutic studies in psoriatic arthritis

Previous Serial Number: None

Principal Investigator: Paul H. Plotz, M.D.

Other Investigators: Alfred D. Steinberg, Ellen Milstone, (CC:DIR), Roger Black (CC:DIR), Naomi L. Gerber, Paul Lucky, and Stephen Paget

Cooperating Units: Clinical Center (Office of the Director)

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

To study the efficacy of two drugs to treat severe arthropathy associated with psoriasis.

Methods Employed:

A randomized, double-blind controlled trial with 3 treatment groups: placebo, azathioprine, and methotrexate, given for 4 months, with objective tests of joint disease at the beginning and end of the period.

Major Findings:

None so far.

Significance to Bio-Medical Research and the Program of the Institute:

This is an attempt to find an effective therapy for psoriatic arthritis with the least danger from drug side-effects.

Proposed Course:

Fifty patients will be studied over the next 2-3 years.

Key Descriptors:

Psoriatic arthritis, cytotoxic drugs, azathioprine, methotrexate, and psoriasis.

Honors and Awards: None

Publications: None

Project No. Z01 AM 41016-03 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The removal of bilirubin and other albumin bound substances from blood

Previous Serial Number: NIAMDD-ARB-16c

Principal Investigator: Paul H. Plotz, M.D.

Other Investigators: Mrs. Kay Gordon, Dr. Bruce Scharschmidt (A:DDB), Dr. Paul Berk (A:DDB), Mr. John Vergalla (A:DDB), and Ms. Jean Waggoner (A:DDB)

Cooperating Units: Digestive Diseases Branch, NIAMDD

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

Substances such as bilirubin that binding tightly to plasma protein cannot be removed by dialysis. We have attempted to remove such substances by employing affinity chromatography on protein - agarose beads.

Methods Employed:

Human albumin is conjugated to agarose: the resulting beads are placed in a column and plasma or whole blood, are passed over the column. Bilirubin or other substances are assayed before and after passage. The bound material is eluted and assayed.

Major Findings:

Agarose-albumin columns can bind in excess of 150 μg bilirubin per gram of wet weight of gel. The BR can be completely eluted and the gel reused. The gel material is compatible with blood and can be used in a perfusion system with living rats. Thyroxine and salicylate bind to such beads too.

Significance to Bio-Medical Research and the Program of the Institute:

Such conjugates may be useful for removing bilirubin and other protein-bound toxic substances from human blood.

Proposed Course:

Further studies will include investigation of the binding of other substances to agarose-protein beads and the preparation of materials safe for *in vitro* use. *In vivo* studies on rats, monkeys and sheep have begun.

Keyword Descriptors:

Bilirubin, jaundice, affinity chromatography, albumin, hemoperfusion, hepatic (liver) failure.

Honors and Awards: None

Publications:

1. Plotz PH, Berk PD, Scharschmidt BF, Gordon JK, Vergalla J: Removing substances from blood by affinity chromatography. I. Removing bilirubin and other albumin-bound substances from plasma and blood with albumin-conjugated agarose beads. J Clin Invest 53:778-785, 1974.

2. Scharschmidt BF, Plotz PH, Berk PD, Waggoner JG, Vergalla J: Removing substances from blood by affinity chromatography. II. Removing bilirubin from the blood of jaundiced rats by hemoperfusion over albumin-conjugated agarose beads. J Clin Invest 53:786-795, 1974.

3. Berk PD, Martin JF, Blaschke TF, Scharschmidt BF and Plotz PH: Unconjugated hyperbilirubinemia: physiologic evaluation and experimental approaches to therapy. Ann Int Med (in press).

4. Plotz PH, Berk PD, Scharschmidt BF, Gordon JK, and Vergalla J: Affinity chromatography for bilirubin removal: development of the method. In Williams, R. (Ed.): *Artificial Support Systems for Acute Hepatic Failure*. London, Pitman (in press).

5. Berk PD, Scharschmidt BF, Plotz PH, Waggoner JG and Vergalla J: Removal of bilirubin from the blood of jaundiced rats by heoperfusion over albumin-conjugated agarose beads. In Williams, R. (Ed.): *Artificial Support Systems for Acute Hepatic Failure*. London, Pitman (in press).

Project No. Z01 AM 41017-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The valence and specificity of rheumatoid factor

Previous Serial Number: None

Principal Investigator: Robert Eisenberg, M.D.

Other Investigators: Paul H. Plotz, M.D. (A:ARB)

Cooperating Units: None

Man Years:

Total: 1 3/4
Professional: 1
Others: 3/4

Project Description:

Objectives:

To determine whether the preference of rheumatoid factor for aggregated gamma globulin is due to new determinants or to multivalent interactions; to determine whether the two binding sites on each subunit of a polymer immunoglobulin are equally able to bind antigen.

Methods Employed:

A purified monoclonal rheumatoid factor was digested to isolate the Fab subunits. These have been shown to bind equally well to manomer and aggregated gamma globulin. A purified mouse IgA myeloma is being studied with small and larger antigens, before and after partial blocking of antibody binding sites with an affinity label.

Major Findings:

The rheumatoid factor preference for aggregated gamma globulin is due wholly to multivalent interaction.

Significance to Bio-Medical Research and the Program of the Institute:

The studies may well be relevant to the role of multivalency of antigen-antibody complexes in stimulating rheumatoid factor responses.

Proposed Course:

The studies on the mouse myeloma protein will be continued.

Keyword Descriptors:

Rheumatoid factor, rheumatoid arthritis, valence

Honors and Awards: None

Publications: None

Project No. Z01 AM 41018-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Production of covalently-crosslinked immune complexes.

Previous Serial Number: None

Principal Investigator: Paul H. Plotz, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Others:	1/4

Project Description:

Objectives:

To produce site-specifically covalently cross-linked antigen-antibody complexes to allow study of certain properties of these complexes.

Methods Employed:

Anti-hapten antibodies are cross-linked with a bivalent affinity-labelling hapten.

Major Findings:

Covalently-linked complexes have been produced and are being studied in detail.

Significance to Bio-Medical Research and the Program of the Institute:

This work may allow studies of the properties of stable immune complexes of various sizes relevant to their role in the pathogenesis of connective tissue disease.

Proposed Course:

Studies will continue

Keyword Descriptors:

Immune complexes, affinity-labelling, cross-linked antibodies.

Honors and Awards: None

Publications: None

Project No. Z01 AM 41019-02 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: **Aspirin** Hepatotoxicity in Patients with Collagen Vascular Disease

Previous Serial Number: NIAMDD-ARB-15c

Principal Investigator: William E. Seaman, M.D.

Other Investigators: Paul H. Plotz, M.D.

Cooperating Units: None

This project was discontinued due to the departure of the principal investigator, June 30, 1974.

Project No. Z01 AM 41020-08 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pathogenesis of Autoimmunity in New Zealand Mice

Previous Serial Number: NIAMDD-ARB-21c

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: Dr. Naomi L. Gerber, Dr. Merrill E. Gershwin, Dr. Adi Gazdar (C-VLL), Dr. Thomas Chused (NDR), Dr. H. C. Morse, III (NIAID), Dr. A. Ahmed (NNMC)

Cooperating Units: NIAID, National Naval Medical Center, NCI-VLL, Georgetown University Hospital and D-OMA Branch.

Man Years:

Total:	1 1/2
Professional:	1
Other:	1/2

Project Description:

Objectives:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they become relatively resistant to tolerance induction, later they spontaneously develop antibodies to RNA and DNA and finally they develop immune complex glomerulonephritis from which they die. Both humoral and cellular immune defects have been reported and are under active investigation.

Cellular cooperation between thymus derived (T) cells and bone marrow derived (B) cells is a recently described feature of humoral immunity. Two distinct T cell subpopulations cooperate in cell mediated immunity. The existence of suppressor T cells has been suggested by the increased antibody response to a thymic independent antigen given with antithymocyte serum (ATS). We have been investigating such suppressor cell function in New Zealand mice in the hope of understanding the pathogenesis of this autoimmune disease.

Methods Employed:

1. Assay for antibodies to RNA and DNA using ^{14}C-labeled nucleic

acids as ligands and the ammonium sulfate precipitation assay. Assay of antibody forming cells to sheep erythrocytes and polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properties of the different cell types.

4. Use of standard skin grafting using allogenic skin to evaluate cellular immune functions in New Zealand mice, and transfer of serum and cell population between young and old New Zealand mice to study the effect in New Zealand mice and other mice.

5. Study of graft versus host disease inducing properties of NZB/W lymphoid cells using the Simonsen spleen weight assay and newborn C3H/He or SWISS mice as recipients.

6. Separation of lymphoid cells functionally (corticosteroid, ATS, anti-0, anti-kappa) and physically (sedimentation).

Major Findings:

1. Suppressor cells have been studied in cellular immunity using the graft versus host reaction (GVH) model. The GVH response measured in newborn C3H mice to 5 x 10^6 NZB/W spleen cells increases between 1 and 6 months and then falls to low levels by 12 months. This age change does not occur in control mice. The high response of 4 1/2 to 6 month NZB/W spleen cells was suppressed by thymocytes or spleen cells from young NZB/W mice. Suppressive activity of thymus was lost gradually between 1 and 4 months of age. Thymocytes from 4 month NZB/W mice could synergize with 12 month NZB/W spleen cells, but could not suppress 4 1/2 month spleen cells, whereas thymocytes from 1 month NZB/W mice could synergize with 12 month NZB/W spleen cells and suppress 4 1/2 month spleen cells. Control strains showed neither suppression nor synergy.

2. Fractionation of spleen cells from young and old NZB/W mice and control strains show no gross differences in fractionation pattern. However, functional differences were observed. We were able to separate spleen cells that have high activity in the mixed lymphocyte reaction, and high responses to mitogens (PHA and Con A). Helper T cells in the response to SRBC were obtained in 2 fractions.

3. Athymic "nude" mice were found to develop spontaneously antibodies to DNA and immune complex glomerulonephritis. These mice were breed on a BALB/c background and were known to be infected with several viruses.

Athymic "nude" mice on a SWISS genetic background and not carrying these viruses did not develop autoimmunity.

4. In vivo treatment with Con A prolonged tolerance to BGG in NZB/W mice, perhaps by stimulating suppressor activity.

5. Both thymosin and Con A as well as non-specific adjuvants such as polynucleotides partially corrected a number of immunologic abnormalities of older New Zealand mice.

6. Complement dependent handling of immune complexes was impaired as NZB/W mice aged.

Proposed Course:

Future studies will attempt to further define and separate different subpopulations of T cells, as well as the relative changes in such populations with age (helper, suppressor, effector, etc.). The characteristics of the separated subpopulations will be defined, particularly those of suppressor cells. The role of genetic and viral factors in the loss of suppressor cells will be investigated in New Zealand mice. The availability of athymic nude mice will allow us to study lack of thymic function in different strains with and without superinfection with different viruses or immunization with nucleic acid antigens. We hope thereby to define the relative contributions of genetic, viral, and immunologic factors in the development of autoimmunity.

Keyword Descriptors: Suppressor cells, Suppressor T cells
Regulation of immune response, Thymic hormone
Fractionation of spleen cells, Con A, Immune complexes

Honors and Awards: None.

Publications:

1. Gershwin, M. E., Chused, T. M., and Steinberg, A. D.: Cytotoxic activity of anti-theta antibody found in the gamma M fraction. Transplantation 18:377, 1974.

2. More, H. C. III., Steinberg, A. D., Schur, P. H. and Reed, N. D.: Spontaneous "autoimmune disease" in nude mice. J. Immunol. 113:686, 1974.

3. Gershwin, M. E., Ahmed, A., Steinberg, A. D., Thurman, G. B. and Goldstein, A. L.: Correction of T cell function by thymosin in New Zealand mice. J. Immunol. 113:1068, 1974.

4. Gerber, N. L., Hardin, J. A., Chused, T. M. and Steinberg, A. D.: Loss with age in NZB/W mice of thymic suppressor cells in the graft versus host reaction. J. Immunol. 113:1618, 1974.

5. Gershwin, M. E., Klingenstein, R. J., Chused, T. M. and Steinberg, A. D.: In vitro and in vivo cytotoxicity to EL-4 sarcoma in New Zealand mice. J. Immunol. 113:1968, 1974.

6. Gershwin, M. E. and Steinberg, A. D.: Effect of Con A on tolerance to BGG in NZB/W mice. Proc. Soc. Exp. Biol. Med. 147:425, 1974.

7. Garner, G. M., Gershwin, M. E. and Steinberg, A. D.: Properties of fractionated spleen cells from NZB/W mice. Cell. Immunol. 15:129, 1975.

8. Gershwin, M. E. and Steinberg, A. D.: Effects of Con A on immunologic abnormalities of NZB/W mice. Int. Arch. Allergy and Appl. Immunol. 48:220, 1975.

9. Miller, G. W., Steinberg, A. D., Green, I. and Nuzzenzweig, V.: Complement dependent alteration in the handling of immune complexes by NZB/W mice. J. Immunol. 114:1166, 1975.

Project No. Z01 AM 41021-07 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Antibodies to Double Stranded DNA and RNA in Spontaneous and Drug-Induced Systemic Lupus Erythematosus and other Diseases

Previous Serial Number: NIAMDD-ARB-20c

Principal Investigator: Dr. Alfred Steinberg

Other Investigators: Dr. Merrill Gershwin, Dr. Naomi Gerber, Dr. Irwin Scher (NNMC), Dr. Herbert B. Lindsley (Walter Reed), Dr. Lawrence Hyman (Walter Reed)

Cooperating Units: National Naval Medical Center

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

Study the characteristics of anti-DNA and anti-RNA antibodies in human and murine lupus. Determine genetic contributions to the immune response to nucleic acid antigens.

Methods Employed:

Farr ammonium sulfate precipitation assay, using single or double stranded C^{14}-KB cell DNA, or C^{14} polyinosinic·polycytidylic acid as antigen. Standard precipitin reaction using labeled and unlabeled nucleic acids. Standard immunization procedures.

Major Findings:

1. Patients with SLE and anti-DNA antibodies were subdivided on the basis of avidity of antibody for DNA. Those patients with high avidity all had renal disease. Those with the lowest avidity did not have renal disease.

2. Precipitating antibodies to DNA occurred in patients with renal disease. Therapy led to an absolute decrease in precipitating antibodies and a relative increase in non-precipitating antibodies.

3. Antibodies to nucleic acids were induced in monkeys by injection with *Trypanosoma rhodesiense*. Similarly, humans infected with *Trypanosoma gambiense* developed such antibodies.

4. Some strains of mice (NZB and ALN) were found to have high responses to immunization with single stranded DNA complexed to a carrier protein. These animals also spontaneously develop antinuclear antibodies late in life. In contrast DBA/2 and BALB/C mice responded poorly to immunization with SSDNA. These mice do not spontaneously develop antinuclear antibodies. Genetic studies of NZB X DBA F_1, mice and backcrosses to the parental strains suggest that a gene on the X chromosome may influence the response to immunization with SS DNA.

5. Synergy was achieved using different immunosuppressive drugs in the induction of tolerance to DSRNA in NZB/W mice.

Significance to Bio-Medical Research and the Program of the Institute:

Genetic studies may reveal one variable in the pathogenesis of antinuclear antibodies as well as explain their occurrence primarily in females.

Qualitative aspects of antinuclear antibodies may explain why some patients develop renal disease and others do not. This enables us to better define patients at risk earlier in the course of their illness which will lead to more vigorous treatment of patients at greater risk and less vigorous treatment of patients at lesser risk.

Proposed Course:

Define the genetics of the antibody response to DNA. Further study the characteristics of antinuclear anitbodies.

Keyword Descriptors: Autoimmunity, antinuclear antibodies,
Anti DNA, anti RNA, genetic control, X-linked
Trypanosomiasis, systemic lupus

Honors and Awards: None.

Publications:

1. Gershwin, M.E. and Steinberg, A.D.: Qualitative characteristics of anti-DNA antibodies in lupus nephritis. Arthritis Rheum. 17:947, 1974.

2. Lindsley, H., Kysela, S. and Steinberg, A.D.: Nucleic acid anti-

bodies in African trypanosomiasis. Studies in rhesus monkeys and man. J. Immunol. 113:1921, 1974.

3. Scher, I., Ahmed, A., Strong, D., Steinberg, A. D., Paul, W. E.: X-linked B-lymphocyte immune defect in the CBA/hN mice. I. Studies of the function and composition of spleen cells. J. Exp. Med. 141:788, 1975.

Project No. Z01 AM 41022-04 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Therapeutic studies in New Zealand mice

Previous Serial Number: NIAMDD-ARB-22c

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: Dr. Naomi Lynn Gerber, Dr. Merrill Eric Gershwin, Dr. Michael C. Gelfand (NIAID)

Cooperating Units: NIAID

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

New Zealand mice, especially the NZB/NZW F_1 spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. These mice are an excellent model for comparing different immunosuppressive drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying specific immunotherapy.

Methods Employed:

1. Comparison of different immunosuppressive drug regimens in natural history of New Zealand mice - antibodies to DNA, proteinuria and survival.

2. Study of thymus grafting and thymic hormone in an attempt at restoring normal regulatory functions and improve the natural history of New Zealand mice.

Major Findings:

New Zealand mice responded to a number of immunosuppressive drug regimens when started at 5 months of age or later in life if the animals did not have significant proteinuria. When they had significant proteinuria they did not respond favorably to the immunosuppressive regimens that were effective earlier in life.

Chlorambucil, an alkylating agent like cyclophosphamide but without the latter's bladder toxicity appears less effective than cyclophosphamide in treatment of disease in NZB/W mice.

Intermittent (every 2 weeks) grafting of thymuses from young NZB mice to syngeneic NZB mice starting at 1 month of age prevented the development of anti-erythrocyte antibodies and reduced lymphoid proliferation.

Significance to Bio-Medical Research and the Program of the Institute:

These studies are designed to provide background experience for clinical studies in which the immunosuppressive drugs may be given in a more effective manner to humans with SLE.

Continued study in mice may provide the background information neccessary for immunological intervention in humans with SLE.

Proposed Course:

Characterization of the mechanisms of immunosuppression by syngeneic young thymocytes.

Keyword Descriptors: Autoimmunity, lupus-like, hemolytic anemia
New Zealand mice, Immunosuppressives
Suppressor cells, T cells, Tolerance

Honors and Awards: None.

Publications:

1. Steinberg, A.D., Gelfand, M.C., Hardin, J.A. and Lowenthal, D.T.: Therapeutic studies in NZB/W mice III. Relationship between renal status and efficacy of immunosuppressive drug therapy. Arth. Rheum. 18:9, 1975.

2. Gershwin, M.E., and Steinberg A. D.: Suppression of autoimmune hemolytic anemia in New Zealand (NZB) mice by syngeneic young thymocytes. Clin. Immunol. Immunopathol. 1975.

3. Hyman, L., Kovacs, K. and Steinberg, A.D.: Drug induced tolerance: Selective induction with immunosuppressive drugs and their synergistic interaction. Int. Arch. Allergy and Appl. Immunol. 48:248, 1975.

Project No. Z01 AM 41023-01 ARB
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Immune Abnormalities in Patients with Systemic Lupus Erythematosus (SLE)

Previous Serial Number: None

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: M. Eric Gershwin, R. O. Morton, Weislaw Glinski (Dermatology Branch, NCI), Charles Brady (Indian Health Service)

Cooperating Units: Dermatology Branch, NCI
Indian Health Service

Man Years:

Total:	3 1/4
Professional:	2 1/2
Other:	3/4

Project Description:

Objectives:

To apply the newer understanding of basic immunologic principles and observations in New Zealand mice (who develop a lupus-like disease) to the study of patients with SLE.

Methods Employed:

1. Study of families of patients with SLE or larger groups of patients with a high incidence of SLE. A battery of immunologic measurements would be made in such patients.

2. Study of lymphocytes from patients with SLE.

a. Distribution on discontinuous gradient.

b. Number and distribution of T, B and null cells and their responses to mitogens or allogeneic cells.

c. Relationship between anti-lymphocyte antibodies in patients with SLE and abnormalities of cell number and/or function.

Major Findings:

1. Particular groups of American Indians have increased annual incidences of SLE. These include Sioux, Crow and Arapaho tribes.

2. Patients with SLE have abnormal distributions of peripheral blood lymphocytes. Those with active disease have a relative decrease in a subpopulation of T cells and an increase in "null" cells.

3. Anti-lymphocytes antibodies occur in association with increased activity of patients with SLE. These antibodies can reduce the number of perpheral T cells and interfere with their function.

Significance:

Populations for further study are being defined and techniques refined for study of humans with SLE. It is hoped that the rewarding observations in the animal model of SLE, New Zealand mice, can begin to be applied to study of the human disease itself.

Proposed Course:

Continue the studies initiated.

Keyword Descriptors: autoimmunity, cell separation, B cells
T cells, null cells,
genetics, Indians

Honors and Awards: None

Publications: None

Project No. Z01 AM 41024-08 ARB
1. Arthritis & Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

Previous Serial Number: NIAMDD-ARB-32c

Principal Investigator: Dr. Elizabeth Neufeld

Other Investigators: Dr. Larry Shapiro, Dr. Ingeborg Liebaers, Dr. Timple Lim, Mrs. Clara Hall, Mr. James Scott

Cooperating Units: Section on Intermediary Metabolism, LBM

Man Years:

Professional:	3
Other:	2 3/4
Total:	5 3/4

Project Description:

Objectives - To study the biochemical defect of genetic disorders of lysosome function, such as mucopolysaccharidoses and mucolipidoses and to use the findings for medical assistance to the patients and their families.

Methods Employed - Two enzymes, α-L-iduronidase and sulfoiduronate sulfatase are purified from human urine by a combination of previously described methods with affinity and chromatography. The enzymatic activity, corrective factor activity, and capture by fibroblasts are determined. Cultured fibroblasts from patients, patients' relatives and normal individuals are used for measurement of enzyme levels, and patient's fibroblasts are used to determine uptake of enzyme.

Serum and lymphocytes of patients and their relatives are examined for iduronate sulfatase and iduronidase activities in order to develop a rapid diagnostic test for the Hunter and Hurler syndromes, respectively.

Major Findings - 1. Two forms of iduronidase (corrective and non-corrective) have been separated from each other. The former is a "high uptake" and the latter a "low uptake" form. There is a difference of molecular weight and ability to bind to a lectin (RCAI). These results confirm the previous suggestion that uptake requires a particular structural feature on the enzyme protein.

2. The assay developed by Lim et al for measuring iduronate sulfatase activity has been adapted to a simple diagnostic procedure for the Hunter syndrome. Using serum, it is possible to distinguish Hunter patients and patients with mucolipidosis II or III from other individuals; using lymphocytes, it is possible to distinguish Hunter and Hurler patients from each other as well as from other individuals. These tests obviate the need for culturing fibroblasts.

Significance to Biomedical Research - 1. The rapid diagnostic test for the Hunter syndrome was developed in response to a demand from families and their physicians to discriminate between the X-linked Hunter syndrome and the clinically similar but autosomal recessive mucopolysaccharidoses. The need for a differential diagnosis is often urgent because of the pregnancy at possible risk.

2. The findings of specificity in the uptake of enzymes by fibroblasts, and of human mutants lacking the high-uptake form, have led to the hypothesis that hydrolytic enzymes must be secreted and pinoytosed in order to localize, in lysosomes, at least in connective tissue. If this hypothesis is correct, it may have implications for the development and delivery of drugs in disorders involving lysosomal functions. These include not only the relatives rare genetic disorders, but also very common conditions such as arthritis.

Proposed Course of Project - 1. Differences between high and low uptake forms of lysosomal enzymes will be sought at the molecular level. Is there a difference in carbohydrate composition? In subunit size or number? Can high and low uptake forms be interconverted?

2. The biochemical defect in mucolipidoses II and III will be sought. This is closely related to (a), since the hydrolyte enzymes of fibroblasts from mucolipidosis patients are of the low uptake variety. Because of the strong possibility that the difference between high and low uptake is in carbohydrate content, special attention will be paid to glycosyl transferances.

3. Tissue specificity of enzyme uptake will be examined.

4. Purification of iduronidase and iduronate sulfatase will be improved, with the goal of eventual enzyme replacement.

5. In cooperation with Dr. Frank Ruddle (Yale) the chromosomal localization of iduronidase, and the location of iduronate sulfatase on the X-chromosome will be determined.

Keyword Descriptors: Hurler syndrome; Hunter syndrome; I-cell disease; lysosomes.

Honors: G. Burroughs Mider Lecture (October 1974)
Dickson Prize of the University of Pittsburgh (December 1974)
Hillebrand Award of the Washington Chemical Society (April 1975)

Publications:

Lim, T. W., Leder, I. G., Bach, G., and Neufeld, E. F.: An assay for iduronate sulfatase (Hunter corrective factor). Carboh. Res. 37: 103-109, (1974).

Neufeld, E. F.: The biochemical basis for mucopolysaccharidoses and mucolipidoses. Progress in Medical Genetics. 10: 81-101, (1974).

Stevenson, R. E., Howell, R. R., and Neufeld, E. F.: Mucopolysaccharidoses, in "Current Diagnosis" (H. F. Conn and R. B. Conn, eds.) Saunders, 1974, 1065-1070.

Shapiro, L. J., Hickman, S., Hall, C. W., and Neufeld, E. F.: Biochemical Studies in Mucolipidoses II and III. March of Dimes - Original Articles Series, in press.

Stevenson, R. E., Howell, R. R., McKusick, V. A., Suskind, R., Hanson, J. W., Elliott, D. E. and Neufeld, E. F.: The iduronidase deficient mucopolysaccharidoses; clinical and roentgenographic features. Pediatrics, in press.

Neufeld, E. F., Lim, T. W., and Shapiro, L. J.: Inherited Disorders of Lysosomal Metabolism Ann. Rev. Biochem., in press.

Shapiro, L. J., and Neufeld, E. F.: The genetic mucopolysaccharidoses and mucolipidoses, in "Basic Neurochemistry" (R. W. Albers, G. J. Siegel, R. Katzman and B. W. Agranoff, eds.), in press.

Neufeld, E. F.: Enzyme Replacement in Genetic Disease in Encyclopedia of Bioethics, (W. T. Reich, ed.) Free Press, New York, in press.

Project No. Z01 AM 41025-04 ARB
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Receptors for IgE on Mast Cell Plasma Membranes

Previous Serial Number: NIAMDD-ARB-23C

Principal Investigator: Henry Metzger, M.D.

Other Investigators : Dr. Chaviva Isersky-Carter, Dr. Daniel Budman, Dr. Guillermo Mendoza, Dr. James Halper, Dr. Reuben Siraganian (D:LMI).

Cooperating Units : Laboratory of Microbiology & Immunology/NIDR

Man Years:

Total :	5 3/4
Professional:	4
Other :	1 3/4

Project Description :

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and, upon exposure to the allergenic antigen, mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells. In addition we wish to understand the mechanism by which the ternary complex of antigen-IgE antibody--cell surface receptor triggers the degranulation of basophilic cells.

Methods Employed:

The basophilic leukemia line continues to grow well in tissue culture although recently the histamine content of the cells has decreased. Recently an IgE producing rat immunocytoma has been obtained from Dr. Hervé Bazin, University of Louvain, Belgium. This tumor secretes large amounts of an IgE for our studies. The tumor can be transplanted in the Lou/M strain of rat.

Major Findings:

Since our previous report the following has been accomplished:

1. Studies on IgE - membrane receptor interaction.
    a. Recent studies indicate that the rate of dissociation of IgE from its cell-surface receptor is even slower than originally estimated when the cells are maintained under optimal conditions where receptor shedding is minimized. Our best estimate of the association constant is 2-3 x 10^{10}.
    b. A limited number of studies have been performed on normal rat mast cells. The kinetics of association and dissociation appear very similar to that observed with the RBL-1 leukemia cells. The cells have 3 to 5×10^5 receptors/cell.
    c. Additional data on the reversibility of the IgE - cell interaction was obtained. It was shown that the IgE can be dissociated from the receptor by exposing the cells to pH 3.0 at 0° for 1-3 minutes. The eluted IgE a is fully capable of binding to a fresh batch of cells and the acid treated cells are viable and fully capable of binding a fresh batch of IgE. Thus the binding reaction has not involved any irreversible change in either of the reactants.
2. Studies on the specificity of the receptor.

    It was previously shown that the receptors bind rat IgE but not human IgE or a variety of other unrelated proteins. Since it is known that a subclass of rat IgG is homocytotropic the capacity of this Ig to inhibit IgE binding was studied. It was found that aggregated IgG but not unaggregated IgG can compete for the IgE binding sites.
3. Preparation of IgE - binding cell fragments.

    Cell free particulate material can be obtained from the tumor cells simply by allowing the cells to incubate at 0° over several hours. More reproducible yields can be obtained by brief sonication with removal of large cell debris by low speed centrifugation. This material retains IgE binding activity the kinetics of association and the specificity being identical to that observed with intact cells. Importantly such preparations can be stored at refrigerator temperatures for months without loss of activity. This material is excellent for studying the in situ properties of the receptor and may be useful as the starting material for isolating receptors.
4. Preparation of plasma membranes.

    Considerable progress has been made in obtaining membranes from the cells in good yields and with a purity comparable to that achieved in other cell systems. These preparations are now being characterized. Ultimately they will be useful for studying the integration of the receptor in the membrane. These materials are also being used in attempts to produce anti-receptor antibodies.
5. Functional studies.

    Our original studies involved attempts to induce histamine release from the tumor cells using antigen--IgE or anti-IgE--IgE stimulation. Although this works well with normal mast cells it failed completely with the tumor cells.

a. Studies with Ionophore: The effects of the Ca^{++} dependent ionophore A23187 were studied. This compound induces non-cytotoxic histamine release from normal mast cells over a very narrow dose range. (At higher doses cytotoxicity is induced) The tumor cells did not respond to the lower doses; only at the higher doses where LDH release indicated cytotoxicity was occurring, was histamine release observed.

b. Ca^{++} flux studies: Since our tumor cells seem incapable of mediating the full chain of reactions starting from antigen - IgE interaction and leading finally to histamine release we have initiated studies on an earlier step - changes in Ca^{++} flux. Mast cells show a markedly enhanced Ca^{++} influx in the presence of ionophore while the RBL cells show a more modestly enhanced Ca^{++} efflux. Comparative studies are underway.

Significance to Bio-Medical Research and the Program of the Institute:

It is of considerable importance to study the behavior of antibodies on the surface of cells in order to more completely understand the mechanism of the immune response. It is on the cell surface that most of the critical steps of the immune response transpire and it is here that one must direct one search for the ultimate mechanisms by which antigen-antibody reactions initiate specific cellular responses. We now have what we believe is the first instance of a system in which a receptor for an immunoglobulin can be studied rigorously. With it we can potentially make significant contributions to our understanding of antigen-induced, antibody-mediated, cellular reactions.

Proposed Course:

1. Characterization of purified membranes from RBL-1 cells.
2. Characterization of receptor *in situ* in cell free particulates.
3. Solubilization and characterization of receptor.
4. Further efforts to define earliest events associated with cell triggering by IgE.

Keyword Descriptors:

IgE, membrane-receptors, mast-cells, basophilic-leukemia, ionophore, calcium, histamine, homocytotropic-antibodies, membranes.

Honors and Awards: None

Publications:

1. Isersky, C., Kulczycki, Jr., A. and Metzger, H.: Isolation of IgE from reagin rich rat serum. J. of Immunol. 112: 1909-1919, 1974.

2. Metzger, H.: Antibody structure and the immune response. In C. F. Fox, (ed.): The Immune System: Genes, Receptors, Signals - Proc. of the 1974 ICN-UCLA Symposium on Molecular Biol., New York, Academic Press, 1974.

3. Carson, D. and Metzger, H.: Interaction of IgE with rat basophilic leukemia cells. IV. Induced changes in surface distribution of receptors. J. of Immunol., 113: 1271-1277, 1974.

4. Carson, D., Kulczycki, A., Jr. and Metzger, H.: Interaction of IgE with rat basophilic leukemia cells. III. Release of Intact Receptors on Cell-Free Particles. J. of Immunol., 114: 158, 1975.

5. Kulczycki, Jr., A. and Metzger, H.: The interaction of IgE with rat basophilic leukemia cells. II. Quantitative aspects of the binding reaction. J. Exp. Med., 140: 1676-1695, 1974.

6. Isersky, C., Metzger, H. and Buell, D. Cell cycle associated changes in receptors for IgE during growth and differentiation of a rat basophilic leukemia cell line. J. Exp. Med., 141: 1147, 1975.

7. Siraganian, R., Kulczycki, A., Jr., Mendoza, G. and Metzger, H.: Ionophore A-23187 induced histamine release from rat mast cells and rat basophil leukemia (RBL-1) cells. J. Immunol., 1975 (In Press).

Project No. Z01 AM 41026-02 ARB
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Quantitation of IgE

Previous Serial Number: NIAMDD-ARB-25C

Principal Investigator: Henry Metzger, M.D.

Other Investigators : D. Carson

Cooperating Units : Dr. Hervé Bazin (University of Louvain-Belgium)
Dr. Kurt Bloch (Harvard Medical School)

Man Years:

Total :	1 1/2
Professional:	1
Other :	1/2

Project Description:

Objectives:

To develop a simple method for quantitating IgE.

Methods Employed:

During the development of a method for purifying rat IgE (Serial No. ARB-24C1973-74) we discovered that rat IgE did not precipitate at $(NH_4)_2SO_4$ concentrations below 40% saturation. This is a property both of rat reaginic and myeloma IgE's. When rabbit anti-IgE becomes bound the IgE becomes precipitable. This has permitted us to develop a simple method for measuring IgE in biological fluids using a Farr type assay with radio-labelled IgE.

Major Findings:

1. As mentioned previously the method has been used to establish IgE levels in rats. In particular we were able to follow the IgE levels after a parasite infection which led to an enhanced response to a conventional antigen (ovalbumin). It was found that the IgE levels rose dramatically ($\sim$ 100X) but that this could not be accounted for by either a specific anti-parasite or anti-ovalbumin response. This suggests that the parasitic infection may lead to a generalized entrancement of 'background' IgE responses.

2. 105 rat IgE myeloma sera were assayed for IgE levels. Simultaneously the relative ability of these sera to inhibit the binding of ^{125}I-IgE to rat basophilic leukemia cells was determined. The hope was that in this way an aberrant IgE or possibly a subclass of IgE might be identified. Only one serum gave discordant results and this is being studied further.

Significance to Bio-Medical Research and the Program of the Institute:

Many laboratories are interested in studying IgE immune responses in rodents. Previously only a cumbersome biological assay was available. The present method should be helpful in this regard.

Proposed Course:

No further studies are planned at this time.

Keyword Descriptors:

IgE, rat IgE, parasite infection, myeloma, basophils, basophilic leukemia, IgE binding.

Honors and Awards: None

Publications:

1. Carson, D., Metzger, H. and Bloch, K. J.: Serum IgE levels during the potentiated reagin response to egg albumin in rats infected with *Nippostrongylus brasiliensis*. J. Immunol., 114: 521, 1975.

2. Carson, D., Metzger, H. and Basin, H.: A simple method for quantitating IgE. J. of Immunol., 1975 (In Press).

Project No. Z01 AM 41027-02 ARB
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1965

Project Title: Structure of IgE in Relation to Biological Function

Previous Serial Number: NIAMDD-ARB-26C

Principal Investigator: Henry Metzger, M.D.

Other Investigators : James Halper, M.D.

Cooperating Units : None

Project Description : Project 26C was discontinued--no useful data were obtained.

The interactions of calcitonin with plasma membranes have been analyzed further in a number of systems. "Specific" (high affinity, low capacity) calcitonin receptors exist in renal and calvarial membrane preparations as shown earlier by competitive binding studies with radioiodinated salmon calcitonin. These investigations have been extended to characterize the effect of the hormone-receptor interaction on the membrane-bound adenylate cyclase enzyme. The renal enzyme was shown to be capable of activation by GMPPNP, an analog of GTP, resistant to phosphatases. GMPPNP also decreased the threshold for activation by calcitonin. Calcitonin analogs activated the adenylate cyclase enzyme with potency ratios similar to those observed in competition for receptor occupancy. Thus differences in analog potency were related largely to effectiveness at the receptor level.

Calcitonin receptors on cultured human lymphocytes show characteristics similar to those on rat renal and calvarial membranes as determined by competitive binding studies. The lymphocyte receptors, however, do not appear capable of modulating the associated adenylate cyclase enzyme. It was concluded that, although calcitonin polypetides have undergone remarkable evolutionary changes in structure and effectiveness, there has been no significant corresponding change in biological nature of calcitonin receptors.
[Drs. Marx, Brown and Aurbach, NIAMDD; Dr. Marc Lippman, MO, NCI]

β-adrenergic Receptor of the Avian Erythrocytes.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes specific stimulation of sodium and potassium transport. This effect is mediated through the adenylate cyclase-cyclic AMP system and can be reproduced by adding exogenous cyclic AMP. The effect of catecholamines on adenylate cyclase, cyclic AMP accumulation and sodium or potassium transport is specific for the levorotatory form of these compounds. Activation in the system by catecholamines is specifically inhibited by 1(+)stereoisomers of β-adrenergic blockers.

The β-adrenergic receptor site has been identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indolyloxy)-2-propanol) is a high-affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to a site on turkey erythrocyte membranes. A series of β-adrenergic agonists and inhibitors compete for this binding site with apparent affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. It was concluded that the stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness reflected validly interaction with the β-adrenergic receptor.

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. A site separate from the catalytic site for ATP involved in regulating adenylate cyclase activity has been identified by determining binding of radioactive guanylyl imidodiphosphate (GMPPNP) to the erythrocyte membrane. The affinities of various purine nucleotides for the "GMPPNP site" parallels closely the apparent affinities of the nucleotides in enhancing isoproterenol-stimulated adenylate cyclase.

Although guanyl nucleotides enhance isoproterenol-activated adenylate cyclase with apparent increase in sensitivity to the agonist, there is nevertheless no change in affinity of the receptor. Neither the affinity nor the number of sites on the β-adrenergic receptor, as determined with iodinated hydroxybenzylpindolol, was influenced by GMPPNP. Activation of adenylate cyclase by the agonist seems to involve agonist-induced guanyl nucleotide-enhanced affinity of the enzyme for ATP.
[Drs. Spiegel, Brown and Aurbach, NIAMDD: Dr. Daniel Hauser, Sandoz, Basel]

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral vein and thence through the major veins to reach the neck where selective samples are taken from each inferior and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10- to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to samples from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers.
[Drs. Spiegel, Brown and Aurbach, NIAMDD; Dr. Doppman, Radiology Department, C.C.; Dr. Potts, Massachusetts General Hospital]

Space Flight Metabolic Studies

Results of the metabolic studies of astronauts in the Skylab series of orbital space flights reported last year on the first two flights of 28 and 59 days have now been nearly completed for the third flight of 84 days. Losses of calcium, virtually equivalent to those in immobilized bedrest, were again seen; the continuing plateau increase in urinary calcium to the end of the 84-day flight with no hint of decline forces the assumption that mineral losses from the skeleton would continue for many months. In this longest space flight, the results of metabolic study were confirmed by significant bone density losses (from the os calcis) in the two astronauts with the greatest calcium excretion. Despite a vigorous exercise regimen, muscle tissue was also lost, indicating that the search, in current studies with which this Branch is cooperating, for effective measures to protect the muscles and skeleton in long space flights is likely to be difficult. [G. Donald Whedon (NIAMDD), Leo Lutwak (UCLA School of Medicine and VA Hospital, Sepulveda, Calif.), Jeanne Reid (NIAMDD), Paul Rambaut, Michael Whittle, Malcolm Smith and Carolyn Leach (all at Biomedical Research Division, Johnson Space Center, NASA, Houston, Texas)]

Serial No. Z01 AM 43,001-10 MDB
1. Metabolic Diseases Branch
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies in Bone Metabolism

Previous Serial Number: NIAMDD-MDB-1 (c)

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Allen Spiegel, James Phang, G. Donald Whedon, S. J. Marx, E. M. Brown and J. Shapiro.

Cooperating Units: Dr. Mones Berman, Mathematical Research Branch, NIAMDD
Metabolism Branch, NCI
S. E. Community Hospital, Washington, D. C.

Man Years:

Total:	.75
Professional:	.25
Other:	.50

Project Description:

Objectives: 1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases. 2) To investigate the response of the parathyroid gland to rapid changes in blood calcium. 3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium. 2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of ^{47}Ca.

Major Findings: Results of the metabolic studies of astronauts in the Skylab series of orbital space flights reported last year on the first two flights of 28 and 59 days have now been nearly completed for the third flight of 84 days. Losses of calcium, virtually equivalent to those in immobilized bedrest, were again seen; the continuing plateau increase in urinary calcium to the end of the 84-day flight with no hint of decline forces the assumption that mineral losses from the skeleton would continue for many months. In this longest space flight, the results of metabolic study were confirmed by significant bone density losses (from the os calcis) in the two astronauts with the greatest calcium excretion. Despite a vigorous exercise regimen, muscle tissue was also lost, indicating that the search, in current studies with which this Branch is cooperating, for effective measures to protect the muscles and skeleton in long space flights is likely to be difficult.

Honors and Awards: Dr. G. Donald Whedon received the 1974 Endocrine Society Ayerst Award.

Keyword Descriptors: Osteoporosis, Mineral Metabolism, Orbital Space Flight, Metabolic Balance, Calcium Metabolism.

Publications:

1. Shapiro, J. R., Moore, W. T., Jorgensen, H., Reid, J., Epps, C., and Whedon, G. D.: Osteoporosis, evaluation of diagnosis and therapy. Arch. Intern. Med. 135: 563-567, 1975.

2. Whedon, G. D., Lutwak, L., Reid, J., Rambaut, P., Whittle, M., Smith, M., and Leach, C.: Mineral and nitrogen metabolic studies on skylab orbital space flights. Trans. Assoc. Am. Physicians 87: 95-108, 1974.

Serial No. Z01 AM 43,002-10 MDB
1. Metabolic Diseases Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Parathyroid Hormone: Physiological Regulation of Secretion; Physical, Chemical, and Immunochemical Properties; Biochemistry; and Structure, and Mechanism of Action.

Previous Serial Number: NIAMDD-MDB-2 (c)

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. S.J. Marx, A.M. Spiegel, E. M. Brown, L.E.Mallette, J.L. Doppman, B. Patten, W.K. Engel, J.T. Potts, Jr., H.T. Keutmann, G.W. Tregear, and H. Niall.

Cooperating Units: Radiology Department, C.C., Intramural Research and Medical Neurology Branch, NINDS, and Endocrine Unit, Massachusetts General Hospital

Man Years:

Total:	4.25
Professional:	1.50
Other:	2.75

Project Description:

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts of bovine parathyroid glands by solvent fractionation, gel filtration and chromatography on carboxymethylcellulose. Purity is assessed by disc gel electrophoresis, ultracentrifugation, immunodiffusion and immunoelectrophoresis, and recovery of the amino- and carboxyl-terminal amino acids. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenylate cyclase, or by a highly sensitive radioimmunoassay. Specific chemical and enzymatic methods are used to cleave the hormone to smaller polypeptide fragments. The polypeptide derivatives are sequentially degraded with exopeptidases and the Edman reaction. Adenylate cyclase is measured by determining conversion of α-^{32}P-labeled ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP is determined by 1) converting the cyclic nucleotide to ATP and measuring the latter with a ^{32}P-ATP exchange reaction or 2) radioimmunoassay.

Major Findings: Mechanism of Action. Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone in vitro activates adenylate cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenylate cyclase in the kidney is found in two different anatomic zones; adenylate cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. Further studies show that virtually all the adenylate cyclase detectable in kidney tissue is found within the renal tubules. This work establishes that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven by indirect in vivo physiological experiments.

Chemistry of Parathyroid Hormone. Application of the "sequenator" method to analysis of parathyroid hormone has allowed deduction of the complete amino acid sequence of bovine parathyroid hormone. The molecule is an 84-amino-acid polypeptide with alanine as the amino-terminal residue and glutamine at the C-terminus. There are two phenylalanines (residues 7 and 34), two methionines (residues 8 and 18), one tryptophan (23) and one tyrosine (43). Previous work from this laboratory showed that the biologically important region of the molecule was located at the amino terminus within a polypeptide smaller than that demarcated by the first aspartate residue. Deduction of the sequence of the molecule allowed chemical synthesis of an amino-terminal biologically active fragment. The tetratriacontapeptide representing residues 1-34 in the hormonal molecule was synthesized by a solid-phase method.

After deblocking and purification the synthetic peptide was tested for biological and immunological activity. The product showed qualitatively all the biological properties, in vivo as well as in vitro, of the native molecule. Thus, the protean effects of the hormone on both renal and skeletal tissue can be accounted for by the same limited region of the molecule. It is also implied that the receptor sites for the hormone must be similar in bone and kidney.

Human Parathyroid Hormone. Parathyroid hormone was purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicated approximately eight amino acid substitutions in the human hormone. The amino acid sequence has been completed for the first 37 amino acids at the amino-terminal end of the molecule (Dr. Hugh Niall, MGH, Boston). Based on this analysis, Dr. Geoffrey Tregear (MGH, Boston) synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully as active *in vitro* as *in vivo* as the hormone isolated from human parathyroid adenomas (Drs. Potts, Niall, O'Riordan, Keutmann and Aurbach).

Efforts are continuing to collect large amounts of human parathyroid tissue from which further quantities of human hormone can be purified. With the highly sophisticated techniques now available for sequence analysis, it is projected that if a further 1 to 2 mg of human parathyroid hormone can be isolated in pure form it will be possible to determine the amino acid sequence in the C-terminal region represented by the remainder of the molecule, residues 38-84.

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization of parathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral vein and thence through the major veins to reach the neck where selective samples are taken from each inferior and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10- to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to samples from the general circulation. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers. [Drs. Spiegel, Brown and Aurbach, NIAMDD; Dr. Doppman, Radiology Department, C.C.; and Drs. Powell and Potts, Massachusetts General Hospital]

Neuromuscular Disease in Primary Hyperparathyroidism. Muscular weakness has been a recognized complication of primary hyperparathyroidism for many years. A prospective study with detailed clinical examination, needle electromyography, nerve conduction studies and histochemical evaluation of quadriceps muscle biopsies was carried out in 16 consecutive patients with primary hyperparathyroidism. Fourteen of the 16 patients were found weak on clinical neurological examination. The weakness was most marked in proximal muscles of the lower extremities and affected muscles were frequently atrophic. Nerve conduction velocity studies were normal. Electromyograms showed brief duration small amplitude motor unit potentials in some patients, and abnormal high amplitude long duration polyphasic potentials, suggesting reinnervation in others. Several patients also showed a decreased number of motor units under voluntary control, suggesting denervation. The major finding on muscle biopsy was atrophy of individual type I and type II muscle fibers, with type II fibers more frequently and more extensively involved. The atrophic fibers were frequently small and angular and stained darkly with NADH-tetrazolium reductase or nonspecific esterase, findings which are specific for denervating disease. Most patients were significnatly better after surgical correction of their disease, although the length of time for resolution of the weakness varied. Further studies of neuromuscular function are underway in subjects with secondary hyperparathyroidism and other forms of metabolic bone disease. Studies of neuromuscular function in subjects with secondary hyperparathyroidism show similar weakness, atrophy and fatigability of proximal muscles. Each of 6 patients with osteomalacia had evidence of neuropathic muscle disease either on electromyography or muscle biopsy or both. [Drs. Mallette and Aurbach, NIAMDD; Drs. Patten, Prince and Engel, NINDS]

Significance to Biomedical Research and Program of the Institute: Same as for July 1968-June 1974.

Proposed Course of Project: Same as for July 1968-June 1974.

Honors and Awards : None

Keyword Descriptors: Parathyroid Hormone, Hyperparathyroidism, Hypoparathyroidism, Neuromuscular Disease, Hormone Receptors, Gastrointestinal Absorption of Calcium, Radioimmunoassay, Parathyroid Carcinoma.

Publications:

1. Aurbach, G. D.: Parathyroid. In Beeson, P. B. and McDermott, W. (Eds.): Cecil-Loeb Textbook of Medicine, 14th Edition. Philadelphia, W. B. Saunders Co., 1975, pp. 1808-1819.

2. Heath, D. A., and Aurbach, G. D.: Ectopic production of parathyroid hormone: a prototype of nonendocrine hormone. In Kryston, J. and Shaw, R. A. (Eds.): Endocrinology and Diabetes. New York, Grune & Stratton, 1975, pp. 215-226.

3. Aurbach, G. D., and Heath, D. A.: Parathyroid hormone and calcitonin regulation of renal function. In Robison, R. R. (Ed.): Kidney International, Vol. 6. New York, Springer-Verlag, 1974, pp. 331-345.

4. Mallette, L. E., Bilezikian, J. P., Ketcham, A. S., and Aurbach, G. D.: Parathyroid carcinoma in familial hyperparathyroidism. Am. J. Med. 57: 642-648, 1974.

5. Marx, S. J., Doppman, J. L., Spiegel, A. M., Wolfe, D. R., and Aurbach, G. D.: Embolization of a mediastinal parathyroid adenoma. J. Clin. Endocr. 39: 1110-1114, 1974.

6. Desbuquois, B., and Aurbach, G. D.: Effects of iodination on the distribution of peptide hormones in aqueous two-phase polymer systems. Biochem. J, 143: 83-91, 1974.

7. Mallette, L. E., Sode, J. E., Marx, S. J., Georges, L. P., and Aurbach, G. D.: Total body retention of orally administered ^{47}calcium in primary hyperparathyroidism. J. Clin. Endocr. 40: 582-583, 1975.

8. Mallette, L. E., Patten, B. M., and Engel, W. K.: Neuromuscular disease in secondary hyperparathyroidism. Ann. Int. Med. 82: 474-483, 1975.

9. Doppman, J. L., Marx, S. J., Spiegel, A. M., Mallette, L. E., Wolfe, D. R., and Aurbach, G. D.: Treatment of hyperparathyroidism by percutaneous embolization of a mediastinal adenoma. Radiology 115: 37-42, 1975.

10. Doppman, J. L., Marx, S. J., Mallette, L. E., Monchik, J., Broadus, A., Spiegel, A. M., Beazley, R., and Aurbach, G. D.: Localization of abnormal mediastinal parathyroid glands. Radiology 115: 31-36, 1975.

11. Monchik, J. M., Doppman, J. L., Earl, J. M., and Aurbach, G. D.: Localization of hyperfunctioning parathyroid tissue. Radioimmunoassay of parathyroid hormone on samples from the large veins of the neck and thorax and selectively catheterized thyroid veins. Am. J. Surg. 129: 413-420, 1975.

12. Heath, D. A., and Aurbach, G. D.: Studies on the binding of ^{125}I-parathyroid hormone to renal cortical membranes. In Talmage, R.V. and Munson, P.L. (Eds.): Calcium Regulating Hormones, Proceedings of the Fifth Parathyroid Conference. Amsterdam: Excerpta Medica, 1975, pp.

Serial No. Z01 AM 43,003-10 MDB
1. Metabolic Diseases Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on the Chemical Nature and Mode of Action of Thyrocalcitonin.

Previous Serial Number: NIAMDD-MDB-3 (c)

Principal Investigators: Drs. S. J. Marx and G. D. Aurbach

Other Investigators: Dr. Edward Brown, MDB, NIAMDD and Dr. Marc Lippman, MO, NCI

Cooperating Units: Medical Oncology Branch, NCI

Man Years:
Total: 1.50
Professional: .75
Other: .75

Project Description:

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal tissue and to lymphoid cells isolated from human and other species or grown in tissue culture.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for binding to receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure. The interaction of the hormone-receptor complex with the adenylate cyclase enzyme is modified by the presence of guanyl nucleotides. In the presence of GMPPNP the threshold for hormonal activation is lowered.

The relative renal responses to calcitonin analogs are similar whether determined by competitive binding or adenylate cyclase activation. Thus potency differences of the analogs tested are largely dependent upon effectiveness at the receptor level. Activity of the adenylate cyclase enzyme of human lymphocytes was not modulated by ambient or bound calcitonin.

Calcitonin receptors with similar characteristics have been identified on cultured human lymphoid cells. Human calcitonin, when compared to salmon calcitonin, competed weakly for the receptor on this human tissue.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure-function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney, bone, and other tissues.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also will be of interest to solubilize calcitonin receptors and further characterize them. Purified lymphoid cell types are being characterized with respect to the density of calcitonin receptors on their surface. The whole cell system also may be applied to evaluate the regulation of receptor metabolism *in vitro* and in human disease states.

Honors and Awards: None

Keyword Descriptors: Calcitonin, Receptor, Adenylate Cyclase

Publications:

1. Marx, S. J., and Aurbach, G. D.: Adenylate cyclase from kidney and bone. In Hardman, J.G., and O'Malley, B.W. (Eds.): *Methods in Enzymology*, Vol. 38, Part C. New York: Academic Press, Inc., 1974, pp. 150-153.

2. Marx, S. J., Aurbach, G. D., Gavin, J. R., III, and Buell, D. W.: Calcitonin receptors on cultured human lymphocytes. J. Biol. Chem. 249: 6812-6816, 1974.

3. Marx, S. J., and Aurbach, G. D.: Calcitonin receptors. In Talmage, R. V., and Munson, P. L. (Eds.): *Calcium Regulating Hormones*, Proceedings of the Fifth Parathyroid Conference. Amsterdam: Excerpta Medica, 1975, pp.

4. Marx, S. J., and Aurbach, G. D.: Renal receptors for calcitonin: coordinate occurrence with calcitonin-activated adenylate cyclase. *Endocrinology*, In press.

Serial No. Z01 AM 43,004-10 MDB
1. Metabolic Diseases Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies on Pseudohypoparathyroidism and Related Disorders

Previous Serial Number: NIAMDD-MDB-4 (c)

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. S. J. Marx, A. M. Spiegel and E. M. Brown

Cooperating Units: None

Man Years:
Total: .50
Professional: .25
Other: .25

Project Description:

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenylate cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudohypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalaci have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radioimmunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: Approximately 100 cases of this disorder have been described in the literature; we have examined 18 of these to date. In normal subjects, parathyroid hormone causes a 30- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease and the sex incidence is approximately 2:1 female-to-male. Several pedigrees have been described wherein pseudophyoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rate of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenylate cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenylate cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone. Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response to TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Cushing's syndrome, basal cell nevous syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Kidney tissue was obtained from a patient with pseudohypoparathyroidism who died suddenly with complications of a massive pulmonary embolus. It was demonstrated in vitro that the membrane fraction of this kidney contained adenylate cyclase activity which was stimulatable by parathyroid hormone. Thus, it was concluded that pseudohypoparathyroidism did not represent a total lack of the renal parathyroid hormone-sensitive adenylate cyclase system.

Significance to Biomedical Research and Program of the Institute: Same as reported for July 1, 1968-June 30, 1974.

Proposed Course of Project: Same as reported for July 1, 1968-June 30, 1974.

Honors and Awards: None

Keyword Descriptors: Pseudohypoparathyroidism, Genetic Disorder, Cyclic 3',5'-AMP, Parathyroid Hormone-Mechanism of Action.

Publications: None

Serial No. Z01 AM 43,005-10 MDB
1. Metabolic Diseases Branch
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Hormone-Mediated Solute Transport: Interaction of Hormones with Cell Receptors; Interrelationship between Biogeneration of Cyclic AMP and Active or Facilitated Transport.

Previous Serial Number: NIAMDD-MDB-5 (c)

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. A. M. Spiegel, E. M. Brown and J. D. Gardner

Cooperating Units: Section on Gastroenterology, Digestive Diseases Branch, NIAMDD

Man Years:
Total: 2.50
Professional: 1.50
Other: 1.00

Project Description:

Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map the function of the receptor against activation of adenylate cyclase; to decipher the putative link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Results are analyzed by double reciprocal, Scatchard, or four-parameter logistic (Rodbard) models utilizing computer programs based on these models. Specific hormone binding is defined in terms of dissociation constant (K_m) of the system. Physiologically significant binding shows K_m's compatible with the dose-response relationship recognized for the hormone at the physiological level.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes a specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (CAMP) system and can be reproduced by adding exogenous CAMP. Stimulation of sodium transport by catecholamines is specifically inhibited by propranolol.

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. 5'-guanylylimidodiphosphate (GMPPNP), an analog of GTP, augments isoproterenol-stimulated adenylate cyclase causing greater activation of the enzyme than with 7 mM sodium fluoride. The effect occurs with a K_m for GMPPNP of 10^{-7} M. A similar K_m was observed for the slight stimulation of the enzyme by the nucleotide in the absence of added hormone. The greatly enhanced stimulation of adenylate cyclase activity by isoproterenol in the presence of GMPPNP is blocked completely by propranolol. The latter does not, however, abolish the stimulation of basal activity produced by the nucleotide. The apparent K_m for isoproterenol activation of adenylate cyclase is decreased one order of magnitude by GMPPNP. GMPPNP binds to the cell membranes with a K_m equivalent to that found for effects on adenylate cyclase activity. Propranolol does not inhibit binding of the nucleotide, but unlabeled GMPPNP and other nucleotides competitively displace labeled GMPPNP from its binding site(s) with apparent affinities comparable to the order of potencies for augmenting catecholamine-stimulated adenylate cyclase. Displacement of GMPPNP from the binding site by GTP or ITP (GTP 100 X as effective as ITP) decreases enzyme activity activated by isoproterenol in the presence of GMPPNP. Thus a high affinity purine nucleotide site is involved in regulating the catalytic function of the adenylate cyclase complex. The apparent affinity of the site for ATP is much lower than for GTP or GMPPNP. This finding indicates that, although ATP is clearly the substrate for the enzyme, it is GTP and not ATP that interacts at the regulatory site (identified as a GMPPNP binding site).

The β-adrenergic receptor site has been identified using a radioiodinated analog of a β-blocker designed for this purpose. Hydroxybenzylpindolol (1-(1-para-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indolyloxy)-2-propanol) is a high affinity β-blocker containing a phenolic group. The latter compound can be radioiodinated to high specific activity and binds specifically to a site on turkey erythrocyte membranes. A series of β- adrenergic agonists and inhibitors compete for this binding site with apparent affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. It was concluded that the stereospecificity of interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological effectiveness reflected validly interaction with the β-adrenergic receptor.

Significance to Biomedical Research and Program of the Institute: There are a number of disease states which probably represent abnormalities of hormone receptors or the coupling between receptor, generation of cyclic AMP and physiological response to the hormone. This project represents the possible development of a model system wherein the connection between receptor binding of hormone, generation of cyclic AMP and transport can be investigated. Diseases such as nephrogenic diabetes insipidus or pseudohypoparathyroidism can better be explained once the inter-connecting links between these cellular events are understood.

Proposed Course of Project: Continuing studies are directed towards isolation of the receptors for particular hormones in receptor cells; solubilization and purification of the receptor; analysis of the inter-connecting substance between binding and adenylate cyclase activation; isolation of the guanyl nucleotide binding protein; identification of the system activated by cyclic AMP in stimulating transport of solute.

Honors and Awards: None

Keyword Descriptors: cyclic 3',5'-AMP; β-adrenergic receptor; sodium transport, potassium transport; erythrocyte membranes; guanyl nucleotides; radioiodinated β-blocker.

References

1. Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Effects of ouabain on catecholamine-stimulated sodium transport in turkey erythrocytes. J. Biol. Chem. 249: 516-520, 1974.

2. Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Stimulation of sodium transport in turkey erythrocytes by cyclic 3',5'-AMP. Endocrinology 95: 499-507, 1974.

3. Spiegel, A. M., and Aurbach, G. D.: Binding of 5'-guanylyl-imidodiphosphate to turkey erythrocyte membranes and effects on β-adrenergic-activated adenylate cyclase. J. Biol. Chem. 249: 7630-7636, 1974.

4. Aurbach, G. D., Fedak, S. A., Woodard, C. J., Palmer, J. S., Hauser, D., and Troxler, F.: β-adrenergic receptor: stereospecific interaction of iodinated beta-blocking agent with high affinity site. Science 186: 1223-1224, 1974.

5. Aurbach, G. D., Bilezikian, J. P., Klaeveman, H. L., and Gardner, J. D.: The beta-adrenergic receptor and cyclic AMP-mediated sodium transport in the avian erythrocyte. In Taylor, S. (Ed.): Endocrinology 1973. London: William Heinemann Medical Books Ltd., 1974, pp. 343-348.

6. Gardner, J. D., Mensh, R. S., Kiino, D. R., and Aurbach, G. D.: Effects of β-adrenergic catecholamines on potassium transport in turkey erythrocytes. J. Biol. Chem. 250: 1155-1163, 1975.

7. Gardner, J. D., Kiino, D. R., Jow, N., and Aurbach, G. D.: Effects of extracellular cations and ouabain on catecholamine-stimulated sodium and potassium fluxes in turkey erythrocytes. *J. Biol. Chem*. 250: 1164-1175, 1975.

8. Aurbach, G. D., Spiegel, A. M., and Gardner, J. D.: β-adrenergic receptors, cyclic AMP, and ion transport in the avian erythrocyte. In Drummond, G. I., Greengard, P., and Robison, G. A. (Eds.): *Advances in Cyclic Nucleotide Research*, Vol. 5. New York: Raven Press, 1975, pp. 117-132.

Section on Gastroenterology, Digestive Diseases Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

During this past year the clinical activities of the Section on Gastroenterology have doubled, primarily due to Dr. Denis McCarthy's becoming a member of this section. In addition to a significant improvement in the quality of the consultations provided by this section, new facilities have been made possible for fluoroscopy and endoscopy. These developments have improved the level of clinical training provided to our clinical associates and have increased our interactions with other clinical investigators at NIH. The increase in personnel has also enabled us to embark on two clinical studies involving patients with ectopic gastrointestinal hormone production: 1) evaluation of H_2-receptor blocking agents in Zollinger-Ellison syndrome, and 2) efficacy of streptozotocin in treating patients with pancreatic cholera. Dr. Harold Roth, extramural director for Digestive Diseases, and Dr. William Bachrach, a guest worker from the Food and Drug Administration, regularly attend our weekly clinical research conference and their extensive clinical experience significantly enhances our clinical research activities.

Our laboratory research activity has also expanded during this past year. In addition to continuing our studies of membrane function, of the mechanism of action of gastrointestinal hormones, and of the role of cyclic nucleotides in regulating gastrointestinal function, this section has begun to make a major commitment to investigate the biochemistry of pancreatic and gastric secretion. This latter development was made possible by having Prof. Jean Christophe and Drs. Patrick Robberecht, Harold Shelby and Leroy Gross in the section as guest workers, and Dr. Shmuel Batzri as an NIH fellow. We have also established formal collaborative relations with Dr. V.L.W. Go (Mayo Clinic) to investigate the immunologic and biologic specificities of cholecystokinin-specific antibodies, with Dr. Josef Fischer (Massachusetts General Hospital) to develop a radioimmunoassay for vasoactive intestinal peptide, and with Professor V. Erspamer (University of Rome, Italy) to explore structure-function relations for various synthetic peptides which are structurally related to cholecystokinin.

(Drs. J. Gardner, D. McCarthy, A. Levy, E. Olinger, R. May, A. Dubois, J. Christophe, P. Robberecht, H. Shelby, L. Gross, S. Batzri and A. Tousimis. T. Conlon, T. Adams and A. Childress.)

Annual Report of the Section on Diseases of the Liver, DDB, NIAMDD
July 1, 1974 through June 30, 1975

The Section on Diseases of the Liver is currently conducting five principal studies. These are summarized briefly as follows:

1. Studies of the Transport of Organic Anions by the Liver

A principal effort in this study involves the development of techniques for quantitating individual steps in the hepatic transport of various cholephilic anions, both in man and in small animals, by employing kinetic studies with BSP, indocyanine green, cholyl-glycine and radiolabeled bilirubin. In the past 2 years kinetic studies in approximately 25 patients with Gilbert's syndrome have clearly demonstrated that this condition represents a heterogeneous population with at least three sets of kinetic defects, two of which are clearly not attributable to defects in glucuronide conjugation. Additional studies in psoriatic patients who have undergone extentive methotrexate therapy strongly suggest that abnormalities in indocyanine green and bile acid kinetics may be sensitive tests of early hepatic dysfunction resulting from methotrexate. Previously, routine liver function tests have been found to be inadequate to detect methotrexate induced hepatic injury and it has been necessary to perform serial biopsies in patients receiving this drug for the therapy of psoriasis. Extensive studies of hepatic organic anion uptake and competition between anions for hepatic uptake in the rat have clearly demonstrated that the uptake mechanism of the liver for BSP, bilirubin, and indocyanine green is saturable, that these anions show mutually competitive yet selective inhibition, and that countertransport can be demonstrated for each of these anions. This data is the most compelling evidence collected to date that the hepatic uptake mechanisms for these organic anions involve a carrier mediated transport process. A further aspect of this study has involved, in the past year, the isolation, purification and radiolabeling of individual conjugates of bilirubin. This project has proceeded to the point where labeled conjugates are now available for physiologic studies in animals. During the next year we hope to achieve adequate purification so that similar studies of the physiology of conjugated bilirubin can be performed in patients.

2. Removal of Organic Anions From Blood by Hemoperfusion

After successfully demonstrating the effectiveness of albumin-agarose affinity chromatography columns for the removal of bilirubin from the blood of jaundiced rats, we have completed a study this year of the use of a similar approach for the management of neonatal jaundice in newborn Rhesus monkeys. While the technique was again found to be highly effective in removing bilirubin from the blood of jaundiced monkeys, the procedure was found to be associated with significant damage to platelets and granulocytes. This is in sharp contrast to our previous studies in rodents in which there was no loss in blood-formed elements. In order to obviate the graulocyte and platelet losses occurring during extracorporeal perfusion, it has been found that regional anticoagulation of the columns with citrate effectively abolishes granulocyte and platelet loss, not only through albumin-agarose columns but through columns of other adsorbents such as activated charcoal and neutral and charged synthetic resins as well. This finding may be of considerable clinical importance in the development of third generation artificial kidneys for which column adsorbents are likely to be an important component.

3. Clinical and Histologic Studies of Vinyl Chloride Associated Liver Disease

During the past year we have had the opportunity to examine and extensively evaluate four patients with vinyl chloride associated hepatic fibrosis and one with angiosarcoma. In collaboration with the Laboratory of Pathology and with the Department of Pathology of the Mount Sinai Medical School, the histologic features of vinyl chloride associated hepatic fibrosis and angiosarcoma at both the light and electron microscopic level have been elucidated. The evolution from hepatic fibrosis to angiosarcoma has also been clearly demonstrated histologically. Extensive effort has been expended in attempting to develop an adequate screening regimen for vinyl chloride workers. To date this has had essentially negative results in that we have shown that all routine tests of liver function and sensitive tests of dye clearance, such as BSP and indocyanine green, may be entirely normal in the presence of extensive hepatic fibrosis. Evaluation of newer tests such as bile acid clearance studies and radioimmunoassay of procollagen peptides is currently being undertaken in collaboration with other units at the National Institutes of Health and the Mayo Clinic.

4. Definition of Immunologic Features and Pathogenetic Mechanisms in Chronic Active Hepatitis and Primary Biliary Cirrhosis

This study involves the modification and the evaluation of normal ranges for a wide variety of tests of all aspects of the immune response including: intradermal skin tests, primary immunization with both protein and polysaccharide antigens, quantitation of T and B cell populations, various serologic tests such as antimitochondrial and antismooth muscle antibodies, and evaluation of reticuloendothelial clearance function employing radiolabeled microaggregated albumin and antibody coated chromium labeled red cells. In addition to the development of a broad experience with the normal ranges for these studies, studies have begun of similar tests in patients with chronic active hepatitis and primary biliary cirrhosis. The results to date in a limited number of patients indicate that there is diminished reticuloendothelial cell clearance of red blood cells coated with IgM antibodies but normal clearance of IgG coated antibodies by patients with both chronic active hepatitis and primary biliary cirrhosis. There is, in addition, a very high incidence of skin test anergy in primary biliary cirrhosis but not in chronic active hepatitis. Highly preliminary studies to date indicate no difference in cell mediated lymphocyte cytotoxicity against Chang liver cell cultures by patients with either of these liver diseases when compared with normal controls.

5. Studies of Copper Metabolism in Man Using Cu^{67} and Cu^{64}

Plasma disappearance curves of intravenously injected radiolabeled Cu^{67}

and simultaneous orally administered radiolabeled Cu^{64} are analyzed by computer in terms of a multicompartmental model of copper metabolism. The computer analysis permits quantitation of various parameters of intestinal copper absorption, hepatic copper uptake, incorporation of copper into ceruloplasmin, copper excretion, and other aspects of internal copper transport. The primary goal of these studies is adequate understanding of normal copper metabolism and the abnormalities in Wilson's disease to permit the determination of a set of kinetic criteria for diagnosis of the heterozygous state for Wilson's disease. In addition, similar studies of copper metabolism will be performed in other disorders, such as primary biliary cirrhosis and prolonged cholestasis, which have previously been shown to be associated with increased copper accumulation in the body.

In addition to the above, which represent the major efforts of the Unit, the following projects are either in developmental stages or are awaiting further methodologic advances prior to the initiation of active research. T These include:

A. Quantitative Determination of Heme Turnover, and of the Origin of Bile Pigments

Validation of measurements of carbon monoxide production and plasma bilirubin turnover as accurate indicators of heme catabolic rate. Determination of the relative contribution of bone marrow and liver to the production of porphyrins and bile pigments, based on the kinetics of labeling of various pigments isolated from blood, urine and feces after simultaneous intravenous administration of bilirubin-^{14}C, glycine-^{15}N, and δ-aminolevulinc acid-2,3-^{3}H (in collaboration with Dr. Samuel Schwartz, University of Minnesota). Studies of the effects of various perturbations such as drug therapy or starvation on heme turnover.

B. Evaluation of the Mechanism of Hepatic Failure Following Bone Marrow Transplantation

Hepatic failure, with an acute Budd-Chiari-like clinical picture has been a major cause of death in the NCI marrow transplant program. To date, detailed review of liver obtained at autopsy from 26 of 30 available cases has detected a unique, and previously unrecognized proliferative lesion of the small hepatic veins, resulting in outflow block. We are currently attempting to correlate this lesion, both retrospectively and prospectively, with other evidence of graph versus host disease, and with preparative radio- and chemotherapeutic regimes in order to determine the pathogenetic mechanism (in collaboration with Dr. Hans Popper).

C. Natural History of Chronic Active Liver Disease Detected on Routine Screening in the Asymptomatic Subject.

A long-term longitudinal study of an increasingly common problem afflicting, among others, at least seven members of the NIH staff (in collaboration with Dr. Paul Plotz and Dr. Leonard Seeff, Washington VA Hospital).

Phoenix Clinical Research Section, Phoenix, Arizona

Investigations into metabolic problems prominent among Southwest American Indians--cholesterol cholelithiasis, diabetes mellitus and obesity--have been conducted during the past year.

Studies relevant to gallstone disease have focused on factors which influence the lipid composition of bile. These include obesity, diet, drugs and female sex hormones.

Obesity was shown to be associated with increased saturation of human bile with cholesterol. This is due to excessive secretion of cholesterol into bile rather than diminished secretion of solubilizing lipids--bile acids and phospholipids. A deficiency of solubilizing lipids, therefore, is not necessary for the formation of lithogenic bile in man. The increased cholesterol saturation and secretion associated with obesity were shown to be reversible by weight reduction. Thus, the prevention of obesity may be useful in the prevention of gallstone disease, and weight reduction may become part of the medical management of cholelithiasis.

An increased synthesis of cholesterol has been shown to accompany obesity in man, and also to accompany the formation of gallstones in hamsters fed a "lithogenic" diet deficient in essential fatty acids. To elucidate the mechanism of increased cholesterol synthesis in this animal model of cholelithiasis, the activity of hydroxymethylglutaryl-CoA reductase was studied in hamsters fed such a lithogenic diet. HMG-CoA reductase is the rate limiting enzyme in cholesterol biosynthesis. Compared to hamsters fed normal chow, the hamsters with gallstones had markedly increased HMG-CoA reductase activity.

It would thus appear that in hamsters fed lithogenic diet, as well as in obese man, supersaturated bile and cholesterol gallstones result in part from increased hepatic synthesis and secretion of cholesterol.

Preliminary studies in man involving dietary manipulation--the feeding of bran and the feeding of refined versus complex carbohydrates--showed no effect on lipid composition of bile.

Previous findings in these and other laboratories that cholesterol desaturation of bile and dissolution of gallstones could be promoted by ingestion of chenodeoxycholic acid have prompted the search for other agents with similar effects. Phenobarbital had been reported by other investigators to increase the bile acid content of animal bile and thereby reduce the level of cholesterol saturation. A preliminary report also suggested that phenobarbital facilitates gallstone dissolution by chenodeoxycholic acid therapy in man. Therefore, extensive studies of the effects of phenobarbital feeding on biliary lipid metabolism in normal human volunteers were completed this year at PCRS. Phenobarbital given for a month or more in daily doses of 3 mg/kg had no consistent or significant effect on biliary lipid composition of secretion, bile acid pool size, bile acid synthesis or fractional catabolic rate, or bile acid composition. These findings make it unlikely that phenobarbital, at least when used alone, will prove to be a useful agent for the prevention or dissolution of cholesterol gallstones.

That female sex hormones influence bile composition is suggested by the female preponderance of gallstones as well as recent reports of excess gallstone disease among users of post-menopausal estrogen and oral contraceptive medication. We found no cyclic monthly variation in bile lipid composition accompanying the hormonal fluctuations of the normal menstrual cycle. The ingestion of oral contraceptive steroids, however, appears to result in regular and significant elevation of the degree of saturation of human gallbladder bile with cholesterol.

Diabetes mellitus, like gallstones, occurs with higher frequency among Southwest American Indians than any other population yet studied. Indian diabetics, like other diabetics, suffer an increased rate of atherosclerosis. We have studied the effects of blood sugar control on cholesterol metabolism in 6 Pima Indian diabetics, and have found increased cholesterol synthesis, increased bile acid synthesis and pool size, and increased plasma cholesterol concentration in the uncontrolled diabetic state as compared to the insulin-treated euglycemic state. This apparent acceleration of cholesterol synthesis may play an important role in the accelerated development of atherosclerosis in diabetics.

Despite a very high prevalence of diabetes and of obesity, both risk factors for the development of atherosclerosis, Pima Indians have a very low prevalence of arterosclerotic heart disease. This would appear to be related to their low levels of plasma cholesterol. The mechanisms responsible for clearance of cholesterol from plasma are being investigated in a study of LDL metabolism currently being initiated. It seems likely that the low prevalence of heart disease and the high prevalence of gallstones in Pima Indians are linked by peculiarities of cholesterol metabolism, the unraveling of which may provide important information for the understanding of both atherosclerosis and cholelithiasis.

Project No. Z01 AM 53000-03 DDB
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Immunophysiology of the Human Small Intestine

Previous Serial Number: NIAMDD-DDB-7(c)

This project was discontinued July 1, 1974.

Publications:

Gebhard, R. L., Falchuk, Z. M., Katz, S. I., Sessoms, C., Rogentine, G. N., and Strober, W.: Dermatitis Herpetiformis. Immunologic concomitants of small intestinal disease and relationship to histocompatibility antigen HL-A8. J. Clin. Invest. 54: 98-103, 1974.

Klaeveman, H. L., Gebhard, R. L., Sessoms, C., and Strober, W.: In vitro studies of ulcerative ileojejunitis. Gastroenterology 68: 572-582, 1975.

Project No. Z01 AM 53001-05 DDB
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Membrane Function

Previous Serial Number: NIAMDD-DDB-9(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Arnold Levy, Jean Christophe, Harold Shelby, Shmuel Batzri and Leroy Gross, NIAMDD; Dr. A. J. Tousimis, Tousimis Research Corp., Inc.

Cooperating Units: Tousimis Research Corp., Inc., Contract No. N01-AM-5-2203

Man Years:

Total:	3.15
Professional:	2.65
Others:	0.5

Project Description:

The broad categories which are included in the project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

During this past year most of our efforts have focused on exploring the functional characteristics of the amino acid transport systems which are stimulated by insulin and on the role of calcium in various secretory processes.

Keyword Descriptors: Membranes, membrane transport, membrane binding

Honors and Awards: None

Publications:

Butler, V. P., Jr., Schmidt, D. H., Watson, J. F., and Gardner, J. D.: Production and properties of digoxin-specific antibodies. Annals N.Y. Acad. Sci. 242: 717-730, 1974.

Project No. Z01 AM 53002-03 DDB
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Gastrointestinal Hormones

Previous Serial Number: NIAMDD-DDB-10(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Jean Christophe, Patrick Robberecht, Harold Shelby, Edward Olinger, Roger May, and Denis McCarthy; Trent Adams and Thomas Conlon, NIAMDD.

Cooperating Units: Diabetes Branch, NIAMDD; Division of Gastroenterology, Mayo Clinic, Rochester, Minn.; and Division of Gastroenterology, Massachusetts General Hospital, Boston, Mass.

Man Years:

Total:	4.55
Professional:	3.05
Others:	1.5

Project Description:

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin and vasoactive polypeptide hormone with their specific membrane receptors.

Clinical investigations are directed toward developing alternative forms of therapy for and elucidating the pathogenesis of disorders characterized by ectopic production of gastrointestinal hormones (e.g. Zollinger-Ellison syndrome and pancreatic cholera).

Keyword Descriptors: Gastrin, cholecystokinin, secretin, vasoactive intestinal polypeptide.

Honors and Awards:

The following papers were selected for presentation: "Initial step in the action of vasoactive intestinal peptide (VIP): Specific binding to plasma membrane receptors" at the National Meeting of the American Society for Clinical Investigation on May 4, 1975; "Actions of gastrointestinal hormones on isolated pancreatic acinar cells" at the Annual Meeting of the American Gastroenterological Association on May 20, 1975; and "The specific binding and action of vasoactive intestinal peptide (VIP) on isolated pancreatic acinar cells" at the International Conference on Pancreatic Function at Titesee, Germany May 22, 1975.

Dr. Jerry Gardner participated in a Symposium on The Chemistry and Metabolism of Gastrointestinal Hormones held October 1974 at the University of Texas Medical Branch, Galveston, Texas. He spoke on "Action of gastrointestinal hormones on the receptor sites of the pancreas."

Publications:

Klaeveman, H. L., Conlon, T. P., Levy, A. G., and Gardner, J. D.: Effects of gastrointestinal hormones on adenylate cyclase activity in human jejunal mucosa. Gastroenterology 68: 667-675, 1975.

Kahn, C. R., Levy, A. G., Gardner, J. D., Miller, J. V., Gorden, P., and Schein, P. S. Pancreatic cholera: beneficial effects of treatment with streptozotocin. New Engl. J. Med. 292: 941-945, 1975.

Gardner, J. G., Conlon, T. P., Klaeveman, H. L., Adams, T. D., and Ondetti, M. A.: Action of cholecystokinin and cholinergic agents on calcium transport in isolated pancreatic acinar cells. J. Clin. Invest. (in press)

Klaeveman, H. L., Conlon, T. P., and Gardner, J. D.: Effects of gastrointestinal hormones on adenylate cyclase activity in pancreatic exocrine cells. In Gastrointestinal Hormones, edited by J. C. Thompson, University of Texas Press, Austin. (in press)

Project No. Z01 AM 53003-02 DDB
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Gastric Emptying

Previous Serial Number: NIAMDD-DDB-11(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Dr. Andre Dubois, NIAMDD; Dr. Mones Berman, NCI.

Cooperating Units: Laboratory of Theoretical Biology, NCI

Man Years:

Total:	1.4
Professional:	1.1
Others:	0.3

Project Description:

Using a dye dilution techique combined with rapid sampling, gastric emptying and secretion are being evaluated *in vivo* in man, dogs, and monkeys. Results obtained from such studies are being used to develop a mathematical model of gastric emptying and to explore the role played by gastric emptying in various clinical disorders such as post-operative ileus, Zollinger-Ellison syndrome, and duodenal ulcer.

Keyword Descriptors: Gastric emptying, mathematical model.

Honors and Awards:

A paper entitled "Altered gastric emptying in Zollinger-Ellison syndrome *not* attributable to gastrin" was selected for presentation at the Annual Meeting of the American Gastroenterological Association on May 22, 1975.

Publications:

None.

Project No. Z01 AM 53004-03 DDB
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cyclic Nucleotide Mediated Functions

Previous Serial Number: NIAMDD-DHDB-12(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Jean Christophe, Patrick Robberecht, Arnold Levy, Edward Olinger, Roger May, NIAMDD; Trent Adams and Thomas Conlon, NIAMDD: Dr. Gerald Aurbach, MDB-NIAMDD; and Dr. A. J. Tousimis, Tousimis Research Corp., Inc.

Cooperating Units: Metabolic Diseases Branch, NIAMDD
Tousimis Research Corp., Inc., Contract No. N01-AM-5-2203

Man Years:
Total: 2.95
Professional: 1.5
Others: 1.45

Project Description:

In vitro systems are being used to characterize the mechanisms by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides. In turkey erythrocytes β-adrenergic catecholamines increase cellular cyclic AMP which in turn stimulates membrane transport of sodium and potassium. In isolated lymphocytes from rat thymus, prostaglandin E_1 and cholera toxin increase cellular cyclic AMP which then stimulates membrane transport of several neutral amino acids. Several gastrointestinal hormones activate adenylate cyclase in plasma membranes prepared from isolated pancreatic acinar cells and also alter various cellular functions; however, at present, it is not clear which, if any, of these functional alterations are mediated by cyclic AMP.

Keyword Descriptors: Adenylate cyclase, guanylate cyclase, cyclic AMP, cyclic GMP, cyclic nucleotide phosphodiesterase, catecholamines.

Honors and Awards:

A paper entitled "The role of cyclic nucleotides in pancreatic secretion" was selected for presentation at the International Conference on Pancreatic Function at Titesee, Germany May 22, 1975.

Publications:

Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Stimulation of sodium transport in turkey erythrocytes by cyclic 3',5'-AMP. Endocrinology 96: 499-507, 1974.

Gardner, J. D., Mensh, R. S., Kiino, D. R., and Aurbach, G. D.: Effects of β-adrenergic catecholamines on potassium transport in turkey erythrocytes. J. Biol Chem. 250: 1155-1163, 1975.

Gardner, J. D., Kiino, D. R., Jow, N., and Aurbach, G. D.: Effects of extracellular cations and ouabain on catecholamine-stimulated sodium and potassium fluxes in turkey erythrocytes. J. Biol. Chem. 250: 1164-1175, 1975.

Gardner, J. D., Jow, N., and Kiino, D. R.: Refractoriness of cation transport in turkey erythrocytes to stimulation by cyclic adenosine 3':5'-monophosphate. J. Biol. Chem. 250: 1176-1185, 1975.

Aurbach, G. D., Spiegel, A. M., and Gardner, J. D.: β-Adrenergic receptors, cyclic AMP and ion transport in the avian erythrocyte. In Advances in Cyclic Nucleotide Research, Vol. 5, Raven Press, New York. (In press)

Aurbach, G. D., Bilezikian, J. P., Klaeveman, H. L., and Gardner, J. D.: The beta-adrenergic receptor and cyclic AMP-mediated sodium transport in the avian erythrocytes. In Endocrinology 1973, William Heinemann Medical Books Ltd., London. (In press)

Project No. Z01 AM 53005-01 DDB
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemistry of the Small Intestinal Mucosa

Previous Serial Number: None

Principal Investigator: Dr. Denis M. McCarthy

Other Investigators: Dr. Roger May, Dr. Jerry Gardner, and Ms. Anne Childress, NIAMDD; Dr. A. J. Tousimis, Tousimis Research Corp.

Cooperating Units: Tousimis Res. Corp., Inc., Contract No. N01-AM-5-2203

Man Years:

Total:	2.0
Professional:	1.0
Others:	1.0

Project Description:

Using in vitro systems, studies are in progress to characterize the subcellular distribution of various membrane-bound enzymes of the intestinal mucosa, as well as their roles in digestive and absorptive function.

Keyword Descriptors: Small intestinal mucosa, digestive enzymes.

Honors and Awards: None

Publications:

Poole, J. R., Mudd, S. H., Conerly, E. B., and Edwards, W. A.: Homocystinuria due to cystathionine synthase deficiency. Studies of nitrogen balance and sulfur excretion. J. Clin. Invest. 55: 1033-1048, 1975.

Hamosh, M., Klaeveman, H. L., Wolf, R. O., and Scow, R. O.: Pharyngeal lipase and digestion of dietary triglyceride in man. J. Clin. Invest. 55: 908-913, 1975.

Project No. Z01 AM 53,500-02 DDB
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of the Transport of Organic Anions by the Liver

Previous Serial Number: 6c

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: James F. Martin, M.D., Bruce F. Scharschmidt, M.D., Allan W. Wolkoff, M.D., John M. Vierling, M.D.

Cooperating Units: None

Man Years:

Total: 2
Professional: 1 1/2
Other: 1/2

Project Description:

Objectives:

1) To study the transport of organic anions from blood through the liver into bile, in order to characterize the biophysical nature of each of the transport steps involved in the overall hepatic excretory system.

2) To develop a capability for quantitating the function of each of these steps in patients with hepatic dysfunction by analysis of kinetic studies of various test substances such as sulfobromophthalein, indocyanine green and radiolabeled bilirubin.

Methods Employed:

Unconjugated bilirubin-^{14}C or -^{3}H is produced biosynthetically in dogs with external biliary fistulae using δ-aminolevulinic acid-^{14}C or -^{3}H as a precursor. After appropriate crystallization and filtration to render the product sterile and pyrogen-free, radiolabeled bilirubin is injected intravenously into patients or animals in order to determine plasma radiobilirubin disappearance curves. Analysis of these curves permits direct calculation of both hepatic bilirubin clearance (C_{BR}: ml/min/kg) and plasma bilirubin turnover (BRT: μmols/kg/day). Further analysis of the disappearance curves in terms of a previously proposed compartmental model of bilirubin metabolism permits direct calculation of such parameters of physiologic interest as hepatic bilirubin uptake rate, the intrahepatic bilirubin concentration, the liver to plasma concentration gradient and the hepatic bilirubin storage capacity. Similar kinetic analyses of plasma disappearance curves of sulfobromophthalein and indocyanine green are also performed.

Major Findings:

Considerable effort has been expended in characterizing the abnormalities of bilirubin metabolism observed in patients with a number of hereditary diseases, such as Gilbert's syndrome and Crigler-Najjar syndrome. Understanding of the defects present in these "experiments of nature" sheds considerable light on normal human hepatic function. Gilbert's syndrome is a common hereditary abnormality characterized by mild unconjugated hyperbilirubinemia which we have shown results from a reduction in hepatic bilirubin clearance. Studies by others have clearly demonstrated that levels of the bilirubin conjugating enzyme, bilirubin UDP-glucuronyl transferase, are reduced in the livers of these patients, but it remains uncertain whether or not this reduction in *in vitro* assays of glucuronyl transferase explains the *in vivo* defect in bilirubin clearance. We have investigated this phenomenon in several ways. First, studies of bilirubin kinetics in patients with Gilbert's syndrome, both before and during treatment with enzyme inducing drugs, have demonstrated that both phenobarbital and glutethimide produce approximately a three-fold increase in hepatic bilirubin clearance in patients with Gilbert's syndrome and, in fact, result in clearances within the normal range. A smaller increase in bilirubin clearance results from similar treatment of normal volunteers. While measurements of UDP-glucuronyl transferase, performed either in our laboratory or elsewhere, in patients with Crigler-Najjar syndrome, Gilbert's syndrome, untreated normal controls and phenobarbital treated normal controls correlate highly with measurements of bilirubin clearance made in corresponding groups, the marked increase in bilirubin clearance observed in patients with Gilbert's syndrome during phenobarbital or glutethimide treatment is not accompanied by a corresponding increase in measurements or glucuronyl transferase activity in liver tissue from similar patients. Thus, although bilirubin clearance becomes normal in patients with Gilbert's syndrome during phenobarbital or glutethimide therapy, this normalization of *in vivo* bilirubin metabolism as measured in our laboratory is not accompanied by a normalization of bilirubin conjugating enzyme levels, as measured in several other institutions. This suggests clearly that the reduction in bilirubin UDP-glucuronyl transferase cannot totally explain the abnormalities in bilirubin kinetics and *in vivo* bilirubin metabolism observed in Gilbert's syndrome.

Although several reviews of Gilbert's syndrome indicate that all other tests of liver function in such patients are normal, we have studied individuals with unconjugated hyperbilirubinemia and normal liver biopsies in whom *in vivo* studies of radiobilirubin kinetics are identical to those of typical Gilbert's syndrome, but in whom there are abnormalities of BSP metabolism. Although the majority of patients with Gilbert's syndrome do have normal BSP metabolism we have characterized two sub-groups, one of which has an apparent defect in the hepatic uptake of BSP as determined by compartmental analysis of BSP disappearance curves. The second sub-group has a defect at a later stage in BSP transport, the nature of which has not been clarified further. Indocyanine green is a water soluble tricarbocyanine dye which is being increasingly used as a test of liver function. In the standard test, 0.5 mg/kg of indocyanine green is injected intravenously and serial samples are obtained for approximately 20 minutes in order to determine the fractional disappearance rate, or half-life, of the injected dye. Studies in our laboratory have indicated that indocyanine green clearance is normal in all patients with Gilbert's syndrome, irrespective of the presence or absence of abnormalities in BSP metabolism, when the standard 0.5 mg/kg dose of indocyanine green is employed. We have now performed studies of the disappearance rates of multiple doses of indocyanine green ranging from 0.5 to 5.0 mg/kg in a large population of normal volunteers and patients with Gilbert's syndrome. In normal individuals and in those patients with Gilbert's syndrome who have normal BSP kinetics, increasing the dose of indocyanine green from 0.5 to 5.0 mg/kg results in only a very small reduction in the fractional disappearance rate of dye from plasma. In contrast, in those patients who have an abnormally slow rate of BSP disappearance from plasma the increase in indocyanine green dose from 0.5 to 5.0 mg/kg is accompanied by a dramatic fall in fractional ICG disappearance rate. A plot of ICG removal rate vs. dose is clearly indicative of saturation kinetics. Michaelis-Menten analysis of these indocyanine green studies demonstrates that there is a sub-class of patients with Gilbert's syndrome in whom the V_{MAX} for indocyanine green uptake into the liver is reduced to 1.5 mg/kg of body weight, compared to a normal mean value of 4.7. The demonstrated abnormalities in indocyanine green and sulfobromophthalein metabolism, both of which are metabolized by pathways which do not involve glucuronide conjugation, clearly demonstrates that at least in some patients with Gilbert's syndrome there are abnormalities of organic anion transport not attributable to defects in bilirubin UDP-glucuronyl transferase.

In another series of studies of bilirubin metabolism, bilirubin turnover and bilirubin clearance were both calculated before and after splenectomy in patients with hereditary spherocytosis. Following splenectomy bilirubin turnover uniformly fell in all patients with hereditary spherocytosis, reaching an average value of approximately 20% of the pre-splenectomy result. In all cases this was associated with a marked prolongation of mean red cell survival and a fall in plasma bilirubin concentration. Comparison of pre-operative and post-operative values for bilirubin clearance demonstrated that in those individuals whose pre-operative bilirubin clearance was normal the marked fall in bilirubin turnover associated with splenectomy produced no change in the rate of hepatic bilirubin clearance. In contrast, in those patients who pre-operatively had a hepatic defect in bilirubin metabolism of the Gilbert's type the marked reduction in bilirubin turnover associated with splenectomy was associated uniformly with an increase in bilirubin clearance. This suggests that the overall pathway for bilirubin removal in patients with Gilbert's syndrome is operating at a level close to saturation. Which particular step in the overall transport process is responsible for this saturation will be determined in future studies.

The relationship between bilirubin turnover, representing the load of bilirubin presented to the liver daily for removal, and hepatic bilirubin clearance, was also examined in a patient with Crigler-Najjar syndrome. In this condition marked hyperbilirubinemia dur to a complete absence of bilirubin glucuronyl transferase results in a constant risk of brain damage.

In one genetic variant of this syndrome hyperbilirubinemia can be alleviated by treatment with enzyme inducing drugs, such as phenobarbital. In another variant, however, there is no response to any pharmacologic modality. Accordingly, in such a patient we explored the possibility that a reduction in bilirubin turnover would result in a proportional fall in plasma bilirubin concentration and reduce the risk of brain damage. A 22-compartmental model of erythrokinetics was developed in order to simulate the effects of chronic phlebotomy on erythrokinetics and bilirubin production. Control studies of bilirubin clearance, bilirubin turnover, red cell lifespan, circulating red cell mass and bone marrow hemoglobin pool were analyzed in terms of this computer model in order to predict the effects of a 500 ml per week phlebotomy. The computer model predicted that phlebotomy would be associated with a marked fall in bilirubin turnover due to the skewing of the red cell age distribution toward young cells. It was expected that this fall in bilirubin turnover would be associated with a corresponding fall in bilirubin concentration in plasma. Accordingly, a patient with Crigler-Najjar syndrome was phlebotomized for a four-month period. In contrast to computer predictions, the plasma bilirubin concentration did not fall. A repeat of the various studies listed above indicated that the computer model's predictions of the effect of phlebotomy on red cell age distribution, red cell survival, circulating and marrow hemoglobin pools and bilirubin tunover, had all been highly accurate. It was found, however, that the fall in bilirubin turnover in Crigler-najjar syndrome was associated with a corresponding fall in bilirubin clearance, cancelling out any possible therapeutic benefit of the decreased rate of bilirubin synthesis. Although these studies produced no therapeutic benefit for the patient, they indicated that the various alternate pathways of bilirubin excretion in patients with Crigler-Najjar syndrome are uniquely dependent on the rate of bilirubin tunover and appear to have a threshhold effect.

Hepatic organic anion uptake was also studied in 350 rats by measuring the initial plasma disappearance rate (V) of intranvenously injected bilirubin, sulfobromophthalein, or indocyanine green. The results of these studies demonstrate a) hepatic uptake of three organic anions can be accurately estimated from plasma disappearance curves; b) hepatic uptake of bilirubin, sulfobromophthalein, and indocyanine green each shows saturation with increasing dose, and plots of V vs dose took the form of rectangular hyperbolae analyzable in terms of Michaelis-Menten kinetics; c) bilirubin, sulfobromophthalein, and indocyanine green show relatively selective, mutually competitive inhibition of hepatic uptake; d) countertransport may be demonstrated for each of these organic anions. These data are all compatible with the existence of carrier mediated transport process for uptake of each of these organic anions.

Significance to Biomedical Research:

Studies of organic anion kinetics in patients with Gilbert's syndrome, Crigler-Najjar syndrome and normal volunteers have led to a marked improvement in our understanding of the excretory function of the liver. In particular, these studies have demonstrated that it is possible to quantitate hepatic function in terms of clearances in the manner analogous to the use of creatinine clearance measurements for quantitating renal function. The ability to accurately measure both bilirubin turunover and bilirubin clearance, the two parameters which determine the plasma biliubin concentration, has made possible a precise functional classification of all cases of unconjugated hyperbilirubinemia. Studies of the sort described above are also leading both to the development of new clinical methods for assessing the function of individual steps in organic anion transport processes and to an improved understanding of the nature of the defects in a variety of disease states.

Proposed Course:

We plan to continue our evaluation of organic anion transport in patients with Gilbert's syndrome. In particular, new approaches to studies of bilirubin kinetics involving the intravenous injection of various loading doses of bilirubin, in addition to tracer doses, will be employed in an attempt to see whether or not those patients with demonstrated abnormalities in the hepatic uptake of indocyanine green and bromosulfophthalein can also be shown to have a corresponding alteration in the hepatic uptake of bilirubin. In addition, the methods currently used for the studies of unconjugated kinetics will be extended to permit studies of the fate of conjugated radiolabeled bilirubin. This will permit the application of these isotope techniques to a variety of more common disease states, including cirrhosis and viral hepatitis.

Keyword Descriptors: bromosulfophthalein, bilirubin, indocyanine green, bile acids, computers, compartmental modeling, organic anion transport

Honors and Awards: None

Publications: None

Project No. Z01 AM 53,501-02 DDB
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Removal of Organic Anions from Blood By Hemoperfusion

Previous Serial Number: 5c

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Bruce F. Scharschmidt, M.D., James F. Martin, M.D.

Cooperating Units: Arthritis and Rheumatism Branch, NIAMDD (Dr. P. Plotz), Animal Resources Branch (Dr. D. Johnson)

Man Years:

Total: 2
Professional: 1 1/2
Other: 1/2

Project Description:

Objectives:

To develop methods for removing from blood those metabolic products which are ordinarily extracted by the liver. The ultimate objective of this project is the development of an artificial support system for use in the management of hepatic failure.

Methods Employed:

To date we have concentrated on the removal of bilirubin from blood. Bilirubin was selected as a test substance because of its extremely high binding to serum proteins, which makes it a particulary difficult substance to remove. We have used the approach of affinity chromatography, using human serum albumin conjugated in high concentration to agarose beads as a prototype of a simple "artificial liver" for the removal of organic anions from the circulation.

Major Findings:

1) Affinity chromatography using albumin agarose beads is a highly efficient means of removing bilirubin, BSP, indocyanine green and a number of other physiologic metabolites from the circulation.

2) In vivo studies in jaundiced rats have demonstrated that one hour of hemoperfusion over an appropriate size column was sufficient to remove virtually the entire circulating plasma bilirubin pool. This is true in both jaundiced Gunn rats, which have hereditary unconjugated hyperbilirubinemia, and in Sprague-Dawley rats, with ligated bile ducts who have conjugated hyperbilirubinemia. Extrapolation from the kinetics of bilirubin removal in rats suggests that three 6-hour hemoperfusions per week would remove the entire bilirubin load produced during this time from a patient with total hepatic failure.

Significance to Biomedical Research:

The ability to support the patient in hepatic failure by means of an artificial system for the removal of waste products would have obvious clinical implications. Even the simple system developed at this point, which removes predominantly bilirubin, has a number of applications. Jaundice in the first week following birth is extremely common and may result from a variety of causes. Although usually benign, severe jaundice during the early neonatal period can result in significant brain damage and the resultant clinical syndrome of kernicterus. Current treatment of neonatal jaundice is limited to phototherapy in mild cases, and exchange transfusion in severe cases. Although phototherapy appears to be safe, long term follow-up studies of its possible side effects are not yet available. In addition, for reasons not understood, it fails to work in a number of cases. Exchange transfusion, even in the best of hands, carries with it an approximately 1% mortality rate and significant morbidity, including the possibility of transmitting blood-borne diseases such as hepatitis. Hence, hemoperfusion of blood of newborns over albumin agarose beads may offer a safe and effective alternative means of treating neonatal hyperbilirubinemia. The availability of umbilical vessels as both sites of entry and exit to the circulation would make the mechanics of hemoperfusion in the newborn period extremely simple. In addition, the potential use of hemoperfusion over albumin agarose columns for the management of diseases characterized predominantly by increases in bilirubin and/or bile salt concentrations, such as Crigler-Najjar syndrome or congenital biliary atresia, is also under study.

Proposed Course:

We plan to evaluate the practical utility of albumin agarose columns for the management of hyperbilirubinemia in the following situations:

A protocol has been designed which permits us to perform hemoperfusion in premature newborn Rhesus monkeys delivered by caesarian section. These animals develop neonatal hyperbilirubinemia similar to that seen in human newborns. This study will permit assessment of the safety and efficacy of hemoperfusion in the newborn period. In parallel with this study we will perform hemoperfusion in newborn lambs. Although sheep do not get neonatal hyperbilirubinemia, newborn lambs are of approximately the same birth weight as human newborns. In addition, because their size is considerably greater than that of newborn monkeys, permitting greater blood sampling, it will be possible to assess in detail various other physiologic effects of hemoperfusion, such as possible depletion of hormones and fatty acids. It is hoped that the successful completion of a detailed evaluation of hemoperfusion in newborn lambs and monkeys will permit a decision as to whether or not this procedure appears to be safe and suitable for trial in human neonatal hyperbilirubinemia. Simultaneously with these studies of neonatal hyperbilirubinemia, which are being performed in collaboration with Dr. Paul Plotz of the Arthritis and Rheumatism Branch of NIAMDD and Dr. David Johnson of the Animal Resources Branch, we will be continuing to work on improvement of the albumin agarose columns both in terms of their flow properties and their bilirubin capacity. As a new aspect of the project we intend to study the properties of a variety of synthetic resins, both charged and uncharged, for the removal of other metabolites which accumulate in the circulation during the course of more generalized hepatic failure. In addition, intense studies are going on to develop a reproducible model of acute, reversible hepatocellular failure in order to serve as a trial for the evaluation of hepatic support systems.

Keyword Descriptors: albumin, agarose, affinity chromatography, bilirubin, jaundice, neonatal monkeys, platelets, white cells, charcoal, resins

Honors and Awards: None

Publications: None

Project No. Z01 AM 53,502-02 DDB
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical and Histologic Studies of Vinyl Chloride Associated Liver Disease

Previous Serial Number: None

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: James F. Martin, M.D., Allan W. Wolkoff, M.D., John M. Vierling, M.D.

Cooperating Units: Laboratory of Pathology, National Cancer Institute (Dr. L. B. Thomas, Dr. T. Triche), Mount Sinai Medical School (Dr. H. Popper)

Man Years:

Total: 2
Professional: 1 1/2
Other: 1/2

Project Description:

Objectives:

To evaluate the natural history, pathology, and appropriate screening procedures for the detection of liver injury occuring in vinyl chloride workers.

Methods Employed:

To date we have had the opportunity to admit a limited number of vinyl chloride workers from several plants to the NIH. The principle methods involved in evaluating their hepatic function includes the application of routine screening tests, scans, peritoneoscopy, percutaneous biopsy, as well as sophisticated kinetic studies of bilirubin, BSP, indocyanine green and bile acid transport in order to evaluate the most sensitive screening procedure for detecting liver injury in this population. In addition to histologic material obtained directly from NIH in-patients, which is evaluated by both light and electron microscopy, histologic materials are sought from other institutions when it can be established that these have been obtained from individuals with occupational history to vinyl chloride exposure.

Major Findings:

1) Vinyl chloride is associated with two distinct types of hepatic injury. These include, first, an unusual pattern of non-cirrhotic hepatic fibrosis and, second, a histologically distinctive type of angiosarcoma. Based on the careful evaluation of specimens obtained from multiple workers, with and without angiosarcoma, it is believed that the fibrotic lesion represents a pre-malignant change.

2) In terms of screening procedures, we have established that routine tests of liver function as well as kinetic tests which depend on the integrity of hepatocytes are highly insensitive in detecting vinyl chloride liver injury because hepatocytic function is spared until very late in this form of liver disease. Since proliferation of collagen is major pathogenetic mechanism in vinyl chloride associated liver injury, we are investigating whether biochemical tests to evaluate collagen metabolism might be more sensitive a screening procedure in this particular condition than routine tests of hepatocellular function.

3) We have established that an appreciable degree of fibrosis may be found in vinyl chloride workers who not only have normal liver function tests but whose liver biopsies appear virtually normal to the light microscope. In several instances, however, the careful examination of such biopsies by electron microscopy has demonstrated increased content of collagen and proliferation of fibroblasts and related cells within the space of discs.

Significance to Biomedical Research:

The recent recognition that vinyl chloride poses a hazard to workers in the vinyl chloride industry represents a major industrial problem which may have considerable implications for a very large population. In addition to 6,500 workers engaged in the preliminarization of vinyl chloride to plastic, an additional 150,000 workers are involved in converting the finished plastic into a final product. These final products are virtually ubiquitous and almost the entire population of the United States is exposed to some degree to such plastics. It has thus become essential that a major biomedical effort be mounted to document the full extent of this problem, which was first recognized less than 18 months ago, and to provide answers to a number of questions such as the total population at risk, the biochemical pathogenesis of the liver injury, the relationship of the liver injury to various other types of neoplasms which have been reported in vinyl chloride workers, the existence of possible safe levels of exposure and so on. To the extent that we have facilities to provide particularly intensive evaluations of individual patients, we are making a contribution to a multi-center effort to evaluate this newly recognized problem.

Proposed Course:

We are continuing to accept new patients as available and to evaluate both their liver function with new tests and various aspects of collagen metabolism as appropriate methodologies can be worked out.

Keyword Descriptors: vinyl chloride, hepatic fibrosis, angiosarcoma, liver biopsy, peritoneoscopy, electron microscopy

Honors and Awards: None

Publications:

Berk, P. D., Martin, J. F., and Waggoner, J. G.: Persistence of vinyl-chloride induced liver injury after cessation of exposure. *Annals N. Y. Acad. Sci.* 246:70-77, 1975.

Thomas, L. B., Popper, H., Berk, P. D., Selikoff, I., and Falk, H.: Vinyl chloride induced liver disease: from idiopathic portal hypertension (Banti's syndrome) to angiosarcoma. *New Engl. J. Med.* 292: 17-22, 1975.

Project No. Z01 AM 53,503-01 DDB
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Definition of Immunologic Features and Pathogenetic Mechanisms in Chronic Active Hepatitis and Primary Biliary Cirrhosis

Previous Serial Number: None

Principal Investigator: E. Anthony Jones, M.D.

Other Investigators: Paul D. Berk, M.D., John M. Vierling, M.D.

Cooperating Units: Immunophysiology Section, Metabolism Branch, National Cancer Institute (Dr. W. Strober, Dr. D. Nelson), Clinical Immunology Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases (Dr. M. Frank, Dr. C. Jaffe)

Man Years:

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives:

To evaluate in detail the immunologic status of patients with chronic active hepatitis and primary biliary cirrhosis with special emphasis on immunologic phenomena currently believed to be of pathogenetic significance in these diseases. The ultimate objective is to identify the major pathogenetic mechanisms in both diseases.

Methods Employed:

Criteria for patient selection include consistent history, physical examination, results of liver biochemical tests, serum non-organ specific antibody titers and hepatic histology. Patients with a history of alcohol abuse or ingestion of hepatotoxic drugs or whose sera are positive for hepatitis B_s antigen are excluded. The study will be limited to HB_sAg negative subjects until adequate safeguards for personnel handling blood products are developed.

The immunologic evaluation includes multiple intradermal antigen tests; primary immunization with keyhole limpet hemocyanin and pneumococcal polysacchride with subsequent keyhole limpet hemocyanin skin testing; quantitation of T and B cell populations using Rosette tests and immunofluorescence; HLA typing; quantitation of serum concentrations of immunoglobins; and functional (hemolytic) assay of complement components. Two additional types of tests specifically evaluate postulated mechanisms of pathogenesis. The first are tests of in vitro cytoxicity of the patient's harvested lymphocytes against Chang human hepatoma cells in culture, using as mitogens the tissue media (control), phytohemagglutin, conconavalin A, and pokeweed mitogen. As a non-specific control, cytoxicity is assessed using EL-4 mouse leukemia cells as a target cell. In each experiment harvested lymphocytes from a normal control are similarly reacted. The second are tests of reticuloendothelial cell function. These tests involve assessing the blood clearance of I^{131} labeled microaggregated human albumin and the patient's Cr^{51} labeled red blood cells which have been selectively coated with either IgM or IgG antibody directed against ABO or Rh antigens.

Using this protocol it is possible to derive information about the function of both the afferent and efferent limbs of a patient's immune response. The assessment of the afferent limb includes data on humoral immunity (primary and secondary responses) and cellular immunity, including T and B lymphocyte function.

Major Findings:

Results to date include the following:

1) In patients with both chronic active hepatitis and primary biliary cirrhosis, no apparent difference appears to exist in cell mediated cytotoxicity of patients' lymphocytes against Chang liver cells and EL-4 cells compared to normal controls.

2) In patients with both chronic active hepatitis and primary biliary cirrhosis, diminished reticuloendothelial cell clearance of IgM coated red blood cells and normal clearance of IgG coated red blood cells compared to controls has been demonstrated.

3) There is a higher incidence of skin test anergy in patients with primary biliary cirrhosis than in patients with chronic active hepatitis and controls. This anergy includes the response to de novo immunization with keyhole limpet hemocyanin.

Significance to Biochemical Research:

Eludication of the pathogenic mechanisms which initiate and sustain the disease process in chronic active hepatitis and primary biliary cirrhosis is required for better diagnostic testing and development of rational therapies. Defined initiating factors exist for those subgroups of chronic active hepatitis associated with HB_sAg or specific drug use wheras initiating factors are unknown for the remainder of patients with chronic active hepatitis and all patients with primary biliary cirrhosis. The mechanisms of pathogenesis leading to sustained disease activity are unknown in both conditions. Current hypotheses favor immunologic mechanism(s). These hypotheses, in part, stem from the high prevalence of abnormal immunologic phenomena in both diseases. The empiric use of adrenal corticosteroids and other immunosuppressive therapies in both diseases is probably a reflection of this view. To date, existing specific hypotheses lack experimental confirmation. In addition, it is not known whether certain immunologic abnormalities found in some subgroups of patients and not in others are epiphenomena or are related to pathogenesis.

The current study provides a comprehensive immunologic characterization in selected patients to define the spectrum of immunologic abnormalities and their relationship to clinical and histologic variables and, in addition, tests of hypotheses of specific pathogenic mechanisms. The significant morbidity and mortality of chronic active hepatitis, the uniform mortality of primary biliary cirrhosis, and the inadequacies of all current therapies in both conditions emphasize the need for information which throws light on the pathogenesis of these diseases.

Proposed Course:

The protocol, as described above, will be continued. Investigations will also be directed toward defining the antigenic specificity of Chang liver cell membrane and developing methods of culturing patients' liver cells obtained by percutaneous biopsy for use as target cells. If appropriate, T-cell suppressor function will be evaluated in collaboration with workers in the Immunophysiology Section of the Metabolism Branch, National Cancer Institute. If adequate safeguards can be instituted, patients with HB_s antigenemia will be included within the protocol.

Keyword Descriptors: chronic active hepatitis, primary biliary cirrhosis, intradermal skin tests, primary immunization, T cells, B cells, lymphocyte cytotoxicity, reticuloendothelial function

Honors and Awards: None

Publications: None

Project No. Z01 AM 53,504-01 DDB
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Copper Metabolism in Man Using ^{67}Cu and ^{64}Cu

Principal Investigator: E. Anthony Jones, M.D.

Other Investigators: Paul D. Berk, M.D., John M. Vierling, M.D.

Cooperating Units: Whole Body Counting Section, Nuclear Medicine Division, Clinical Center (Dr. R. Aamodt, Mr. W. Rumble), Department of Neurology, George Washington University (Dr. S. O'Reilly)

Man Years:

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

Objectives:

To evaluate quantitatively the metabolism of copper in health and specific diseases associated with abnormalities of copper metabolism.

The ultimate goals include a comprehensive understanding of normal copper metabolism, and an evaluation of the significance of specific abnormalities of copper metabolism in patients with hepatolenticular degeneration and their relatives and patients with primary biliary cirrhosis and other disease states.

Methods Employed:

1) Studies using ^{67}Cu alone. After the intravenous injection of ^{67}Cu serial measurements are made of whole body radioactivity (using a whole body counter), hepatic radioactivity (using a scintillation probe), and radioactivity in whole blood, red cells, plasma, ceruloplasmin, urine and feces. With the exception of hepatic radioactivity, these measurements are made for a period of 13 days. Plasma ceruloplasmin-bound ^{67}Cu determination involves the removal of non-ceruloplasmin-bound ^{67}Cu from plasma samples by passage of these samples down small columns of activated charcoal. Plasma non-ceruloplasmin-bound ^{67}Cu are determined by subtracting radioactivity in ceruloplasmin from that in whole plasma. The curves of total body, hepatic, whole blood, red cell, plasma ceruloplasmin and plasma

non-ceruloplasmin radioactivity are defined and the total excretion of radioactivity in urine and feces calculated.

2) Studies using ^{67}Cu and ^{64}Cu. Studies are also being conducted in which ^{67}Cu is being administered by intravenous injection and ^{64}Cu is being administered simultaneously by mouth. The subsequent protocol is the same as for studies using ^{67}Cu alone except that both ^{67}Cu and ^{64}Cu radioactivity are measured in all experimental specimens. In measuring ^{67}Cu and ^{64}Cu simultaneously an appropriate correction is made for the radioactivity due to ^{64}Cu which cannot be excluded when measuring ^{67}Cu. The plasma disappearance curve of non-ceruloplasmin-bound ^{67}Cu is used to correct the plasma non-ceruloplasmin-bound ^{64}Cu curve for losses employing deconvolution and integration procedures on a digital computer. This enables the total ^{64}Cu which passes from the gut lumen to the plasma to be calculated. This quantity less the amount of the administered ^{64}Cu which does not appear in feces gives an estimate of the biliary secretion of ^{64}Cu and hence of the enterohepatic circulation of this metal.

Major Findings:

1) The ranges of whole body ^{67}Cu retention for normals and homozygotes and heterozygotes for hepatolenticular degeneration have been defined.

2) There was appreciable incorporation of ^{67}Cu into ceruloplasmin in normals and a similar degree of incorporation in heterozygotes for hepatolenticular degeneration. In contrast, incorporation of ^{67}Cu into ceruloplasmin in homozygotes for hepatocellular degeneration was minimal.

3) After administering ^{67}Cu intravenously and ^{64}Cu orally to normal volunteers, plasma curves of non-ceruloplasmin-bound ^{67}Cu and ^{64}Cu have been defined and the feasibility of studying the enterohepatic circulation of copper using these two isotopes has been established.

Significance to Biomedical Research:

In certain disease states marked variations in the copper content of plasma and various organs are known to occur. However, the mechanisms by which copper homeostasis is maintained in health and disturbed in disease are pooly understood. Increasing attention has been focused on copper metabolism in hepatolenticular degradation since the refutation of the concept that low ceruloplasmin concentration is primarily responsible for manifestations of the disease. Recent work has demonstrated diffuse deposition of copper in the liver in primary biliary cirrhosis raising the possibility that abnormal copper metabolism may contribute to the pathogenesis of this disease, the etiology of which is at present unknown.

An important potential cause of deranged copper metabolism is a defect in the intestinal absorption of the metal. For example, it is possible that increased copper absorption may contribute to the increased hepatic copper which occurs in hepatolenticular degeneration and chronic cholestasis. Alternatively, copper absorption may be impaired in diseases of the small intestinal mucosa. Lack of reliable methods, variability in hepatic copper uptake and the presence of an enterohepatic circulation for copper have hitherto prevented acquisition of reliable data on absorption.

Using the double radionuclide technique outlined, it is possible to quantitate the intestinal absorption and biliary secretion of copper in human subjects and hence to determine whether there is a derangement of intestinal absorption or biliary secretion of copper in specific disease states.

Proposed Course:

To achieve the stated objectives it is necessary to study more normal subjects, patients with hepatolenticular degeneration and their relatives, patients with primary biliary cirrhosis and other patients who may have abnormal copper metabolism.

The protocol, as defined above, will be continued. Initially, emphasis will be placed on studying a sufficient number of volunteers to permit normal ranges for variables of copper metabolism to be established. In homozygotes for hepatolenticular degeneration, analysis of the data will focus on the nature of abnormalities of copper metabolism in relation to the manifestations of the disease, while in heterozygotes for this disease analysis of the data will be largely directed towards defining defects in copper metabolism which can readily be measured and which enable the heterozygote state to be distinguished from normal subjects. Further investigations in man may involve direct analysis of bile copper excretion using a multiple lumen enteric tube. It is proposed to conduct similar studies using both ^{67}Cu and ^{64}Cu in the rat.

Keyword Descriptors: copper, intestinal absorption, Wilson's disease,
primary biliary cirrhosis

Honors and Awards: None

Publications: None

Project No. Z01AM 54,001-03DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cholesterol and Biliary Lipid Metabolism in Obesity

Previous Serial Number: NIAMDD-DDB-13(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn Bennion

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	0.5
Others:	0.5

Project Description:

Objectives: To determine whether obesity is associated with changes in bile which would predispose to gallstone formation, and if so, to determine the mechanisms responsible for these changes.

Methods Employed: Bile lipid composition, bile lipid secretion rates, bile acid kinetics and pool size, bile acid composition have been, and are being, determined in obese subjects before and after weight reduction.

Major Findings: Weight reduction resulted in significant reduction in the level of saturation of human bile with cholesterol, significant reduction in the rate of secretion of cholesterol and significant reduction in bile acid pool size.

Significance: Obesity evidently predisposes to cholesterol gallstone formation through the formation of supersaturated bile. The production of supersaturated bile in obesity is not a consequence of a deficiency of bile acids, but rather of an excessive secretion of cholesterol. Weight reduction results in a decreased level of saturation of bile with cholesterol.

Proposed Course: The study is almost complete and will soon be prepared for publication.

Keyword Descriptors: obesity, cholelithiasis, cholesterol, bile, bile acids, isotope dilution

Honors and Awards: None

Publications: None

Project No. Z01AM 54,002-03DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Effects of Bile Acid Feeding on Biliary Lipids and Cholesterol Metabolism in Non-Indian Subjects

Previous Serial Number: NIAMDD-DDB-14(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn Bennion

Cooperating Units: None

Man Years:

Total:	0.1
Professional:	0.0
Others:	0.1

Project Description: This project has been discontinued.

Keyword Descriptors: chenodeoxycholic acid, cholelithiasis, bile, bile acid, cholesterol

Honors and Awards: None

Publications:

Adler, R., Bennion, L., Duane, W., Grundy, S.: Effect of Low Dose Chenodeoxycholic Acid Feeding on Biliary Lipid Metabolism. Gastroenterology. 68: 326-334, 1975.

Project No. Z01AM 54,003-03DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Effect of Diabetic Control on Cholesterol Metabolism

Previous Serial Number: NIAMDD-DDB-15(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn Bennion

Cooperating Units: University of California at San Diego School of Medicine

Man Years:

Total	0.4
Professional:	0.2
Others:	0.2

Project Description:

Cholesterol balance, bile acid pool size and turnover rate, bile lipid composition and hepatic secretion rates of cholesterol, bile acid and phospholipid are being measured in six Pima Indians with maturity-onset diabetes mellitus and uncontrolled hyperglycemia (blood sugars over 250). These measurements are then repeated after normoglycemia (blood sugars around 100) has been achieved with insulin therapy.

Methods Employed: Cholesterol balance is determined during several weeks of hyperglycemia, and at the end of this period bile studies are performed. After regulation of blood sugar with insulin, the above studies are repeated. Cholesterol balance is determined by chemical methods using non-absorbable markers given by mouth. Fecal steroids are quantitated by gas-liquid-chromatography. Isotope dilution methods are employed in the determination of bile acid pool size and turnover rate. Bile lipid composition is determined by standard chemical procedures on bile obtained by duodenal siphonage.

Major Findings: Preliminary data suggests that the synthesis of cholesterol is increased in the uncontrolled diabetic state (as compared with the normoglycemic state) in these subjects, and that bile acid pool sizes are expanded, resulting in decreased saturation of bile with cholesterol.

Significance: The findings suggest that cholesterol synthesis is increased in the diabetic hyperglycemic state. If this conclusion is confirmed, it may provide an explanation for the increase in atherosclerosis among diabetics.

Proposed Course: The patient studies now in progress will be completed and the results prepared for publication.

Keyword Descriptors: diabetes mellitus, cholesterol, bile acid, arteriosclerosis, cholelithiasis, blood sugar, insulin

Honors and Awards: None

Publications: None

Project No. Z01AM 54,004-03DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Critical Micellar Concentration of Human Biliary Mixed Micelles

Previous Serial Number: NIAMDD-DDB-16(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. William Duane

Cooperating Units: None

Man Years:

Total:	0.0
Professional:	0.0
Others:	0.0

Project Description:

Objectives: The bile salt-lecithin mixed micelle serves the important function of solubilizing biliary cholesterol; however, formation of this mixed micelle is poorly understood. We have investigated formation of this mixed micelle by studying the intermicellar bile salt concentration (IMBC) in equilibrium with the mixed micelle which allows estimation of the critical micellar concentration (CMC) of the mixed micelle.

Methods Employed: Equilibrium dialysis has been used to measure the IMBC.

Major Findings: In all systems studied the IMBC was directly proportional to B/P; however, in pure bile salt-lecithin systems, the IMBC for taurocholate-lecithin micelles was much higher than the IMBC for taurochenodeoxycholate-lecithin micelles. Results for whole bile fall in between these two extremes. Extrapolation of the IMBC to zero B/P yielded critical micelle concentrations in these systems. The mean CMC of five bile samples was about 0.5 mM while the CMC of the taurochenodeoxycholate-lecithin mixed micelle was about 0.6mM and the CMC of the taurocholate-lecithin mixed micelle was about 3.0 mM.

Significance: The fact that the CMC of the taurocholate-lecithin mixed micelle is so much higher than the CMC of the taurochenodeoxycholate-lecithin micelle suggests that taurocholate has considerably less micelle-forming potential than taurochenodeoxycholate. This observation may explain the reduced capacity of taurocholate to solubilize cholesterol and the ability of chenodeoxycholate to dissolve gallstones in man.

Proposed Course: This project is now essentially complete. It is presently being prepared for publication by Dr. Duane, who has left NIAMDD.

Keyword Descriptors: bile, bile acid, micelles, equilibrium dialysis, cholesterol, phospholipid

Honors and Awards: None

Publications: None

Project No. Z01AM 54,005-03DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Hepatic HMG CoA Reductase in Genetically Obese Rats

Previous Serial Number: NIAMDD-DDB-17(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Eunice Flock

Cooperating Units: Center for Disease Control, Phoenix Laboratories

Man Years:

Total:	0.4
Professional:	0.2
Others:	0.2

Project Description:

HMG CoA reductase is the rate-limiting enzyme in the synthesis of cholesterol and the amount of this enzyme in the liver increases rapidly after feeding. Peak values have been found by others at midnight in rats fed ad lib. The elevated levels of blood cholesterol in genetically obese rats could be due either to increased production of cholesterol or decreased conversion to bile acids.

In a preliminary study of 5 months duration, ending in June 1973, twelve genetically obese rats and twelve of their non-obese siblings were fed chow diet ad lib in a room with a dark period from 4:30 pm to 8:00 am. The rats were killed by decapitation between 10:00 am and 11:00 am and thus presumably during fasting. The livers were assayed for cholesterol content and HMG CoA reductase activity. The concentrations of cholesterol and triglycerides in blood were also measured.

Major Findings: The assay of this enzyme in our hands has been greatly improved during a study of this enzyme in the hamster. It would be worthwhile to complete the study as outlined in the summary of proposed work of June 30, 1974 when time and the availability of the Zucker obese rats permit.

Keyword Descriptors: obesity, HMG CoA reductase, cholesterol, rats

Honors and Awards: None

Publications: None

Project No. Z01AM 54,006-03DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Alcohol Metabolism in American Indians and Caucasians: A Comparative Study of Rates, Mechanisms and Adaptive Capacity

Previous Serial Number: NIAMDD-DDB-18(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn Bennion

Cooperating Units: Indiana University Medical Center

Man Years:

Total:	0.8
Professional:	0.4
Others:	0.4

Project Description:

Objectives: Recent studies have shown genetic and racial differences in the rate of alcohol metabolism. This project is intended to examine racial variations in alcohol metabolism in Southwestern American Indians and Caucasians.

Methods Employed: The rate of alcohol metabolism was determined by oral alcohol tolerance test. Blood alcohol was determined by gas-solid chromatography.

Major Findings: No significant difference between Caucasians and Indians in the average rate of alcohol metabolism was found.

When the data are analyzed in terms of history of alcohol intake, both Caucasians and Indians show an increase in rate of alcohol metabolism associated with a history of heavy intake.

Significance: These studies do not confirm the earlier report of more rapid metabolism of alcohol in Caucasians than in Indians, and therefore cast doubt on the inference that the high rate of alcoholism among Indians may be related to a low rate of clearance of alcohol from the blood.

Proposed Course: Studies completed and submitted for publication.

Keyword Descriptors: alcohol, Indian, Caucasian, race

Honors and Awards: None

Publications: None

Project No. Z01AM 54,007-02DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of Short-Term Fasting on Biliary Lipid Composition, Bile Acid Pool Size, and Bile Acid Synthesis

Previous Serial Number: NIAMDD-DDB-21(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Ronald Ginsberg and Lynn Bennion

Cooperating Units:

Man Years:

Total:	0.8
Professional:	0.4
Others:	0.4

Project Description:

Bile acid and cholesterol metabolism during fasting have been studied in subhuman primates; however, the effects of fasting on sterol metabolism in man remain poorly understood. We have used isotope dilution techniques to measure bile acid synthesis and pool size in the fed state and again after a four-six day fast in six subjects. In addition the lipid composition of gallbladder bile has been examined in these subjects during the fed and the fasted state.

Methods Employed: Isotopic cholic acid was administered orally and gallbladder bile obtained on the four subsequent mornings. After the fourth bile sample, each subject fasted for five-six days. On the evening of the fourth fasting day cholic acid labelled with a different isotope was administered and gallbladder bile obtained the two subsequent mornings. Total bile acid pool size and cholic acid pool size were calculated from the specific activity in bile obtained the first control day and again the first fasting day. This method of pool size determination has been validated previously in this laboratory and is precise to ±2.6%. Cholic acid synthesis and fractional catabolic rates were calculated by standard methods for the fed state. Calculation of these parameters for the fasting state was achieved by assuming a single pool model for cholic acid and rapid changes in synthesis and fractional catabolic rates at the beginning of the fast. Gallbladder bile lipid composition and individual bile acid composition were measured by standard techniques.

Major Findings: Total bile acid pool and cholic acid pool were either slightly reduced or unchanged by fasting. Cholic acid synthesis was reduced by a factor of three or four in all subjects during fasting. The fractional catabolic rate of cholic acid was reduced by fasting in all subjects. Fasting reduced the molar percent of cholesterol (relative to the solubilizing lipids, bile acids and lecithin) in all but one subject. Individual bile acid composition did not show any consistent change during fasting.

Proposed Course: Investigative work has been completed. The results are now being submitted for publication.

Keyword Descriptors: cholelithiasis, cholesterol, bile acid, fasting

Honors and Awards: None

Publications: None

Project No. Z01AM 54,008-02DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Rapid Determination of Bile Acid Pool Size

Previous Serial Number: NIAMDD-DDB-22(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Lynn Bennion and Ronald Ginsberg

Cooperating Units: None

Man Years:

Total:	0.2
Professional:	0.0
Others:	0.2

Project Description: This project has been discontinued:

Keyword Descriptors: bile acid, bile, isotope dilution, bile acid kinetics

Honors and Awards: None

Publications:

Duane, W., Adler, R., Bennion, L., Ginsberg, R.: Determination of Bile Acid Pool Size in Man: A Simplified Method with Advantages of Increased Precision, Shortened Analysis Time and Decreased Isotope Exposure. J.Lipid Res. 16: 155-158, 1975.

Project No. Z01AM 54,009-02DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Effect of Phenobarbital Upon Biliary Lipid Secretion and Metabolism in Normal Subjects

Previous Serial Number: NIAMDD-DDB-19(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Ronald Ginsberg and Marc Garnick

Cooperating Units: None

Man Years:

Total:	2.0
Professional:	1.0
Others:	1.0

Project Description:

Recent studies have shown that, in the primate, phenobarbital, a known potent inducer of liver microsomal enzymes, had the following effects on biliary lipid metabolism and composition: 1) increased bile flow rates 2) increased bile salt and phospholipid secretion with a concomitant decrease in cholesterol secretion by the liver 3) a consequent decreased cholesterol content in bile relative to bile salts and phospholipids 4) augmented bile salt synthesis rate and bile salt pool size in animals with intact enterohepatic circulations 6) enhancement of the maximal bile salt secretion rate in animals with total biliary fistulae.

Studies in human subjects with cholesterol gallstones have shown that phenobarbital may decrease bile cholesterol content and have a useful synergistic role with chenodeoxycholic acid in the dissolution of cholesterol gallstones.

Methods Employed: Normal volunteers are given a tracer dose of radioactively labelled cholic acid, and a total bile acid pool size, catabolic rate of cholic acid, and synthesis of cholic acid are determined from the subsequent fall in specific activity of cholic acid over the next four days. The relative bile composition with respect to cholesterol, bile salts, and phospholipids is also determined. Hourly secretion rates of cholesterol, bile salts, and phospholipids are also determined by the previously described method of Grundy and Metzger. All studies are performed on the same patients before and after the ingestion of phenobarbital for two months at a dose of 3 mg/kg per day; each patient serves as his own control.

Controls will be studied in the same manner with the substitution of a placebo for phenobarbital.

Major Findings: Phenobarbital did not consistently significantly alter any of the biliary lipid metabolism parameters measured.

Significance: Phenobarbital, at least when used alone, would not appear to be a useful oral agent for prevention or dissolution of cholesterol gallstones.

Proposed Course: A final report is now being prepared to submit for publication.

Keyword Descriptors: phenobarbital, cholesterol, cholelithiasis, bile, bile acids

Honors and Awards: None

Publications: None

Project No. Z01AM 54,010-02DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: HMG CoA Reductase in Livers of Hamsters during Gallstone Formation

Previous Serial Number: NIAMDD-DDB-20(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Eunice Flock and Ronald Ginsberg

Cooperating Units: Center for Disease Control, Phoenix Laboratories

Man Years:

Total:	1.0
Professional:	0.5
Others:	0.5

Project Description:

The development of cholesterol gallstones in young hamsters fed a high-glucose diet deficient in essential fatty acids has been studied for more than 20 years by Dam and many other investigators. That the formation of the gallstones may be due to increased synthesis of choleserol is suggested by the greater incorporation of ^{14}C acetate into cholesterol in hamsters after intraperitoneal injection in hamsters on the lithogenic diet compared to the rice-starch diet (Jensen and Dam) and by the greater incorporation of radioactive acetate into cholesterol by liver slices of hamsters on the high-glucose diet (Muroya and coworkers).

Objectives: The purpose of this investigation, which began in 1973 was to determine the effect of the lithogenic diet on HMG CoA reductase, the rate-limiting enzyme for the synthesis of cholesterol. In addition the concentration of cholesterol in serum, liver, hepatic microsomes and carcass of hamsters on the lithogenic diet has been compared with that in hamsters on the chow diet. The composition of bile acids in gallbladder bile has also been determined.

Methods Employed: Young male golden Syrian hamsters were kept in a room artificially illuminated for 12 hours during each 24 hour period. They were fed either the "lithogenic" diet of Tanimura or the Wayne Lab-Blox diet. Feeding schedules were (a) ad lib or (b) food was deposited by automatic hoppers in each cage at the beginning of the dark period and residual food pellets removed at 4, 6 or 12 hours later.

The hamsters were anesthetized with CO_2. Blood was obtained by cardiac puncture prior to surgery. The gallbladder was removed and examined for stones. The livers were excised and microsomes isolated for assay of HMG CoA reductase. Cholesterol concentrations were measured in serum, liver, carcass and hepatic microsomes. The relative amounts of cholic, chenodeoxycholic and deoxycholic acid in gallbladder bile were determined by gas liquid chromatography.

Major Findings: Cholesterol gallstones were found in 35 of 51 hamsters which had been on the lithogenic diet for 26-49 days. None of the hamsters on the chow diet developed gallstones.

No consistent increase in enzyme activity was found in chow fed hamsters between 4 and 6 hours after the onset of the dark period (postprandial). Elevated values for HMG CoA reductase were found, however, at this time of feeding, in hamsters as early as the second day on the lithogenic diet. Mean values for the activity of this enzyme in the lithogenic group was 428 ± 50 ($n = 37$) pmoles/min/mg microsomal protein ±SEM in contrast to 36 ± 4 ($n = 35$) in hamsters on the chow diet.

Cholesterol did not accumulate in liver on carcass of the lithogenic fed hamsters but the concentration of serum cholesterol increased. Hepatic microsomal cholesterol concentrations were lower in the chow-fed hamsters than in the lithogenic group.

There was a shift in the composition of bile acids in gallbladder bile in hamsters on the lithogenic diet. A higher percentage of chenodeoxycholic acid and a lower percentage of cholic acid were found in lithogenic diet fed hamsters than in chow fed animals.

Significance: The increased HMG CoA reductase activity in lithogenic diet fed hamsters is indicative of increased cholesterol synthesis. This could lead to increased cholesterol secretion in bile and thus to lithogenic bile and finally to gallstone formation.

Proposed Course: Further studies of hamsters on the lithogenic diet will include measurement of serum insulin by radioimmunoassay. It is known that the increase in HMG CoA reductase activity which occurs in rats following feeding is due to synthesis of the enzyme, and that insulin is required for the normal diurnal rise in the activity of this enzyme. It is possible that the lithogenic diet with its high percentage (63.5%) of glucose stimulates the release of more insulin than does the chow diet.

Keyword Descriptors: cholelithiasis, HMG CoA reductase, cholesterol,
gallstones, hamster, bile acids

Honors and Awards: None

Publications: None

Project No. Z01AM 54,011-02DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Effects of the Menstrual Cycle and Oral Contraceptives Upon Biliary Lipid Composition

Previous Serial Number: NIAMDD-DDB-23(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Lynn Bennion, Ronald Ginsberg and Marc Garnick

Cooperating Units: None

Man Years:

Total	1.0
Professional:	0.4
Others:	0.6

Project Description:

The composition of gallbladder bile in relation to the varying hormonal phases of the normal menstrual cycle has been determined in 6 women through a total of eight cycles. The composition of gallbladder bile in 22 women taking oral contraceptive steroids is also being measured, and compared to that when they are not taking oral contraceptives.

Methods Employed: Gallbladder bile is collected by duodenal aspiration following an overnight fast. Gallbladder contraction is stimulated by intraduodenal infusion of amino acids. Lipids in gallbladder bile are measured by standard chemical procedures, and the percent saturation of bile with cholesterol is calculated.

Major Findings: No regular or significant variation of bile composition with the menstrual cycle has been detected; however, the ingestion of exogenous contraceptive steroids results in a regular and statistically significant increase in the degree to which gallbladder bile is saturated with cholesterol in these healthy young women.

Significance: These findings suggest that 1) epidemiologic surveys of bile composition can be conducted without regard to the timing of the menstrual cycle, and 2) ingestion of oral contraceptives may increase the risk of developing cholesterol gallstones, and 3) the female preponderance of gallstones may be mediated by an effect of the female sex steroids on the lipid composition of bile.

Proposed Course: The studies are being completed and will soon be submitted for publication.

Keyword Descriptors: female, sex, bile, gallstone, cholelithiasis, bile acid, cholesterol, menstrual cycle, estrogens, progestins, oral contraceptives

Honors and Awards: None

Publications: None

Project No. Z01AM 54,012-01DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Effect of Dietary Fiber (Bran) upon Biliary Lipid Composition

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Ronald Ginsberg and Marc Garnick

Cooperating Units: None

Man Years:

Total:	0.2
Professional:	0.1
Others:	0.1

Project Description:

Recent observations have linked an increased incidence of cholesterol gallstones with the consumption of diets high in refined carbohydrate content and low in bulk constituents. Diets high in bulk (fiber) contents have been associated with higher total bile acid pool sizes in man as well as an increased proportion of chenodeoxycholic acid as compared to diets with high refined sugar content. The purpose of the present study is to investigate the effect of a diet supplemented with a high bran (fiber) content on the cholesterol content of gallbladder bile.

Methods Employed: Normal human subjects will have gallbladder bile collected before and after a 4-8 week period of high fiber (bran) consumption. The bile will be analyzed for the relative proportions of cholesterol, bile acid, and phospholipid content as well as the proportions of individual bile acid species comprising the total bile acid pool.

Major Findings: Two subjects have completed the study and have shown no significant changes in bile lipid composition after the period of bran feeding.

Proposed Course: Two other subjects are now on the study; if no changes are found the study will be terminated.

Keyword Descriptors: bran, cholelithiasis, cholesterol, bile acid

Honors and Awards: None

Publications: None

Project No. Z01AM 54,013-01DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evaluation of Combined Oral Contraceptives on the Oral Glucose Tolerance Test and Cortisone Glucose Tolerance Test in Normal Women

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Stephen Aronoff, Richard Hamman, Peter Savage, Phillip Gorden, and Jesse Roth

Cooperating Units: Phoenix Indian Medical Center

Man Years:

Total:	0.3
Professional:	0.2
Others:	0.1

Project Description:

Objectives: The effect of oral contraceptives on carbohydrate metabolism has stimulated much interest, but in spite of the substantial amount of research the results have been, in some instances, contradictory.

We have noticed a relationship between combination-type oral contraceptives and abnormal cortisone glucose tolerance tests while studying normal subjects without family history of diabetes under Project Number J01.82-A-74 (Evaluation of the Prediabetic State in the Pima Indians-Caucasian Controls). Out of the 31 subjects with normal oral GTT's, we found 6 subjects with abnormal cortisone GTT's, 5 of whom were on a combined oral contraceptive. (All 5 of these subjects' oral GTT's were indistinguishable from normal subjects as well as 4 of 5 of their IVGTT's.) In no instance has a subject on oral contraceptives had a normal cortisone GTT.

The study was designed to verify and further investigate our observation of abnormal cortisone GTT's in subjects on oral contraceptives in the presence of normal GTT's.

Methods Employed: Female volunteers (with a negative family history for diabetes) who are planning to start to stop oral contraceptives are being studied. An oral glucose tolerance test and a cortisone glucose tolerance test are performed on each woman while on oral contraceptives and again after stopping them or vice versa.

Major Findings: Not yet available.

Significance: This study should clearly delineate the effects of oral contraceptives on glucose and insulin response during the Oral Glucose Tolerance Test and the Cortisone Glucose Tolerance Test.

Proposed Course: Eleven subjects have been partially or fully tested. Data will be collected on 8 to 10 more subjects before analysis.

Keyword Descriptors: Oral Glucose Tolerance Test, Cortisone Glucose Tolerance Test, oral contraceptives

Honors and Awards: None

Publications: None

Project No. Z01AM 54,014-01DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

NIH-PHS
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Low Density Lipoprotein Metabolism in Southwestern American Indians

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Marc Garnick, Terry Langer, Lynn Bennion, and Ronald Ginsberg

Cooperating Units: Hospital of the University of Pennsylvania
Philadelphia, Pennsylvania

Man Years:
Total: 1.0
Professional: 0.7
Others: 0.3

Project Description:

Objectives: The extremely low incidence of atherosclerotic cardiovascular disease (ASCVD) in Southwestern American Indians compared to non-Indian populations and the finding that patients with type II hyperlipoproteinemia have a prolonged biological half-life (T 1/2) and decreased fractional catabolic rate (FCR) of low density lipoprotein (LDL) have stimulated interest in this protein's metabolism in full blooded Southwestern American Indians. Although the incidence of obesity and diabetes mellitus are greatly increased in some Indian populations, the American Indian is able to maintain a lower serum cholesterol than that of the general population. We are studying the turnover rate of LDL to elucidate the significance of this factor and its potential role in exerting a protective effect in predisposing to ASCVD.

Methods Employed:

1. Patients are initially placed on a low cholesterol, high polyunsaturate: saturated fat diet for three weeks.

2. LDL is isolated from plasma via multiple step, density gradient ultracentrifugation.

3. The LDL is iodinated with ^{125}I via the iodine monochloride method.

4. The preparation is tested for immunologic purity (agarose gel electrophoresis) pyrogens and sterility according to U.S.P. standards.

5. The radio-labelled preparation is injected intravenously; plasma decay curves and urinary excretion of radioactivity are measured daily to determine the T 1/2 and FCR, LDL synthetic rate.

Major Findings: Three full blooded Southwestern American Indians are in the process of completing their studies. Forthcoming results will be available shortly. The T 1/2 of one study patient's LDL is definitely more rapid than that reported for ASCVD-free non-Indian subjects studied at other institutions (2.74 days vs. 3.6 days).

Significance: The establishment of a difference in T 1/2 and FCR of LDL in Indian populations may be a crucial factor in explaining the low incidence of ASCVD in these people, despite the presence of other factors, which, in non-Indian populations, tend toward the development of cardiovascular disease. Positive results may call for a critical reexamination of current diet and drug therpy designed to lower serum cholesterol values in an attempt to prevent coronary artery disease. Investigation of methods to alter LDL metabolism may be a more basic preventative approach.

Proposed Course: Ten to twelve people will be studied in the next year.

Keyword Descriptors: low density lipoprotein, cholesterol, protein iodination, atherosclerotic cardiovascular disease, ultracentrifugation, American Indians

Honors and Awards: None

Publications: None

Project No. Z01AM 54,015-01DD
1. Digestive Diseases
2. Phoenix Clinical Res.
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Determination of the Age of Onset of Lithogenic Bile Among Southwest American Indians

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Lynn Bennion, Stephen Aronoff, Marc Garnick

Cooperating Units: Argonne National Laboratories

Man Years:

Total:	0.2
Professional:	0.1
Others:	0.1

Project Description:

Objectives: The lipid composition of gallbladder bile, bile acid pool size and blood sex hormone levels will be determined in 10 prepubertal Pima Indians of each sex, 10 post-pubertal (age 14-15) Pima Indians of each sex, and 10 young adult (age 18-21) Pimas of each sex.

Methods Employed: Bile will be obtained by duodenal siphonage after stimulation of gallbladder contraction by intraduodenal infusion of amino acids. Bile acid pool size will be measured by determining the mass ratio of deuterated chenodeoxycholic acid to native chenodeoxycholic acid in the bile twelve hours after the intragastric administration of dideuterochenodeoxycholic acid. Determination of bile acid composition by gas-liquid chromatography will permit calculation of total bile acid pool size from the chenodeoxycholic acid pool size determined as above by mass-spectrometry. Bile lipid composition will be determined and expressed as percent saturation with cholesterol. Changes in bile composition and bile acid pool size will be correlated with changes in blood level of sex hormones, age, pubertal history, body size, and sex.

Major Findings: None

Significance: These studies should disclose whether the secretion of abnormally saturated bile is acquired in the pubertal or teen-age period among Pima Indians, or whether it is present in childhood. They should also disclose the relationship between the onset of lithogenic bile and the incidence of cholelithiasis, since the incidence of the latter has been carefully determined in this population. They should also elucidate the role of the sex hormones in the production of lithogenic bile, as well as the importance of bile acid pool size in the production of lithogenic bile in this population. Comparison of body size measurements and of age to bile acid pool size may yield important data regarding the regulation of bile acid pool size.

Proposed Course: The project will be started after Tribal consent has been obtained.

Keyword Descriptors: Pima Indians, cholelithiasis, cholesterol, bile acid, stable isotopes

Honors and Awards: None

Publications: None

ANNUAL REPORT - 1974-1975

CLINICAL ENDOCRINOLOGY BRANCH

In 1974, in recognition of the increasing strength of the intramural program in diabetes research, the Diabetes Section was separated from the Clinical Endocrinology Branch and became the Diabetes Branch. Dr. Jesse Roth, Chief of the Section since just after it was established, became first Chief of the new Branch.

The clinical endocrinology training program has become well established and successful. It has produced an increased interchange between the endocrinology and metabolism groups within our own and other Institutes, and has been instrumental in attracting outstanding applications for our Clinical Associate staff.

Dr. Robbins was elected President of the American Thyroid Association for 1974-75. Exchange visits with laboratories in Paris, Kyoto, Rome and Naples took place, and research workers from Italy, France, Japan, Israel and Switzerland participated in research at CEB in Bethesda.

I. Thyroid Biochemistry

A. Thyroid Cell Membrane and the Mechanism of TSH Action

Initiation of thyrotropin (TSH) action requires binding to receptors on the cell surface. Studies on beef thyroid membranes showed that binding is relatively stable, and that most modulators of TSH action act on the adenylate cyclase of the plasma membranes at a stage beyond binding. The relationship between binding and activation is complex and not yet elucidated. TSH itself consists of α and β subunits. The latter subunit, which is free of TSH contamination, can bind to the TSH receptor and activate thyroid function *in vitro*, but its effect is much lower than that of intact TSH. The α subunit also had a small effect, but this may be the result of contamination with TSH. Thus the β subunit contains the determinants necessary for TSH action whereas the α subunit enhances this activity through a conformational change. Luteinizing hormone (LH) also binds to the TSH receptor, but is much less effective in stimulating biologic activity. (Wolff, Kohn)

In a study of the effect of nucleotides on adenylate cyclase activation, it was shown that 5'-guanylylimidodiphosphate (Gpp(NH)p) is highly effective in thyroid as well as in a number of other endocrine tissues. The nucleotide not only enhances the activity of TSH on thyroid, ACTH on adrenal, etc., but also is intrinsically active. Since it does not stimulate adenylate cyclase in E. coli, it would appear to be a useful probe for investigating the mechanism of hormone and nucleotide action in eukaryotic cells. (Wolff, Rodbell)

A soluble adenylate cyclase obtained from Bordatella pertussis is under study as a model for the cyclase in thyroid. In addition, the regulation by the cyclase of toxic products released from this organism is under investigation. (Wolff, Hewlett)

Activation of protein kinases may be the mechanism through which cyclic AMP exerts its action on the cell. Thyroid protein kinase activity has been demonstrated in microsomal and nuclear fractions as well as in fractions enriched in plasma-membranes. In bovine thyroid slices, TSH, by increasing the intracellular concentration of cAMP, activates protein kinase activity. The cAMP receptor subunit of the kinase is capable of being phosphorylated by the catalytic subunit of the intact molecule. This autophosphorylation may be an important regulator of the dissociative response of protein kinase to cAMP. (McClung, Rall).

B. Iodoproteins

Elucidation of the subunit composition of thyroglobulin has proved to be very difficult, apparently because of hydrolytic nicks in the polypeptide chain occurring during storage in the colloid. It has now been shown that guinea pig thyroglobulin exposed to reduction and alkylation displays much less heterogeneity than does thyroglobulin from other species and thus permits clearer demonstration of the TG subunit structure. Molecular weights of the 3 components determined by equilibrium sedimentation are 295,000, 210,000 and 110,000, and their amino acid compositions are similar. The heaviest species predominated in low iodine thyroglobulin, whereas the lightest predominated when iodination was heavy. The subunit composition of thyroglobulin is apparently affected by the degree of iodination but the mechanism by which this happens is still unknown. (Haeberli, Rall, Edelhoch, Salvatore)

Certain transplantable thyroid tumors in rats have been shown to contain abnormal thyroglobulins, iodoproteins which react with anti-TG antibodies but differ from TG in physical properties. Since these proteins could not be recovered from an $(NH_4)_2SO_4$ precipitate or be purified by DEAE-cellulose chromatography, affinity chromatography was investigated as a method for their isolation. The abnormal thyroglobulins were shown to bind to columns of anti-TG immunoglobulin coupled to agarose. Elution proved to be difficult, but was achieved with 0.05 M glycine-NaOH, pH 11. The eluate retained TG-like immunoreactivity, had a sedimentation rate of $\sim$8 S in sucrose gradient ultracentrifugation and appeared as one excluded and one retarded peak in gel filtration on Bio-Gel A5M. Further characterization of these abnormal iodoproteins are in progress. (Izumi, Robbins, Cahnmann)

C. Thyroxine Synthesis

Intramolecular coupling of two diiodotyrosine (DIT) residues in thyroglobulin is believed to be the mechanism by which thyroxine is synthesized in vivo. This reaction should give rise to a new amino acid derived from the phenylalanine side chain of one of the DIT residues and remaining in the peptide chain, but this presumed by-product has never been identified. Various linear copolymers of tyrosine with a single other amino acid have been investigated for their ability to form thyroxine upon enzymic iodination. Interestingly, only one of several copolymers investigated yielded detectable amounts of thyroxine: a copolymer of tyrosine and lysine which gave a 15% yield of thyroxine. Experiments are planned to identify the amino acid arising from the "lost" phenylalanine side chain. (Cahnmann, Nunez, Pommier)

D. Thyroid Hormone Secretion

The secretory process of many types of cells appears to involve microtubules, which are polymers of a repeating subunit, tubulin. The chemical and physical properties of this protein have been investigated. Binding of 8-anilino-1-naphthalene sulfonate occurs on a site different from the colchicine-binding site. The binding ratio is 1:1, and both compounds become fluorescent when bound. Details of the fluorescence properties have been worked out. Although the microtubules are found within the cytoplasm, it was also shown that colchicine-binding activity could be solubilized from membranes of thyroid and brain by detergent, and that the properties of membrane-bound and soluble tubulin were similar. (Wolff, Bhattacharyya)

Li^+ is one of the very few compounds which blocks thyroid iodine secretion and also blocks the adrenal secretory response to ACTH. Preliminary evidence indicates that Li^+ produces this effect by stabilizing microtubules, and further verification is in progress. (Wolff)

E. Thyroxine-Protein Interactions

Several new electrophoretic methods have been applied to the identification of minor thyroxine-binding proteins in human plasma. Confirmation has been obtained that lipoproteins are involved and four such proteins have been shown to bind thyroxine: α_1-lipoproteins, β-lipoprotein and two pre-β-lipoproteins. (Lewallen)

One of the major thyroxine-binding proteins in human plasma is prealbumin (PA), a protein which also transports Vitamin A through an interaction with retinol-binding protein. Since the structure of the PA molecule is almost completely known, it has been used to investigate the T_4-protein interaction on a molecular level by the method of affinity labeling. The first experiments were done with dimethylaminonaphthalenesulfonate (DNS), which binds covalently to PA within the T_4-binding site. Study of the hydrolytic products of dansylated PA showed that DNS was attached to Lys-15, an amino acid residue located within the channel formed by the four identical PA subunits. Apparently one Lys in each of the two binding sites islabeled. Experiments are now under way with bromoacetyl-T_4. This molecule also binds covalently to Lys, and identification of the specific residue is in progress. (Cheng, Cahnmann, Wilchek, Robbins, Ferguson)

The protein which transports about 3/4 of the serum thyroxine is the thyroxine-binding alpha globulin, TBG. Affinity chromatography on T_4-Sepharose has been used to purify TBG and a radioimmunoassay for this protein has been developed. In normal humans the TBG level in plasma is 1.4 ± .4 mg/dl. When compared to the thyroxine-binding capacity of TBG measured by zone electrophoresis, this indicates that TBG has a single binding site for the hormone. The assay has been applied to sera from patients with hepatocellular carcinoma, since abnormalities in serum protein production are known to occur. TBG was elevated in some sera but normal in many, and there was no correlation with α-fetoprotein elevations. Similar procedures have been used for purification and measurement of TBG in rhesus monkeys, and are being applied to the study of TBG synthesis in monkey liver cells. (Gershengorn, Glinoer, Robbins)

F. Treatment of Thyroid Carcinoma

The use of lithium and of thyrotropin releasing hormone (TRH) in the management of functional thyroid carcinoma is under investigation. In one patient with highly functional metastases, Li^+ was shown to slow significantly the rate of ^{131}I loss from tumor. This would be expected to increase tumor radiation from a therapy dose, while at the same time decreasing bone marrow radiation which is derived largely from circulating organic radioiodine. However, ^{131}I therapy in this patient produced severe bone marrow toxicity. The potential of lithium to improve the therapeutic toxic ratio of ^{131}I requires further cautious exploration.

TRH administered in large doses by mouth to patients with thyroid carcinoma has been shown to produce a persistent rise in serum TSH levels over a 14 day period. This should permit ^{131}I uptake stimulation by endogenous TSH without the necessity to make the patient hypothyroid. In highly functional tumors, Li^+ could then be added to the regimen to prevent feedback inhibition of TSH secretion by the thyroid hormone secreted by the tumor. (Robbins, Gershengorn)

II. Polypeptide Hormones

A. Structure

Self association of the glycoprotein hormones, and their interaction with 8-anilino-1-naphthalenesulfonate (ANS), was studied in order to learn more about the hydrophobic sites on the surface of these hormones, and to evaluate apparent cooperativity in the self association reactions. Both human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH) undergo limited self-association dependent on the concentration of ANS. In the case of hLH, binding of ANS to a single site per dimer leads to negative cooperativity in the association reaction, which proceeds only to dimer formation. In the case of hCG, addition of ANS first leads to formation of tetramers, with 4 strong binding sites, and then to dimers, which have 4 sites of lower affinity. ANS binding in this case exhibits positive cooperativity. A general model was developed for these interactions, which may have a role in biological control. (Ingham, Edelhoch)

B. Secretion and Metabolism of Glycoprotein Hormones and their Subunits

Antisera specific for the common subunit and the specific β subunits of these hormones have been developed and used to investigate the regulation of subunit and complete hormone production. Free subunits of TSH and LH appear in the plasma after stimulation by the appropriate hypothalamic releasing factor, prior to appearance of the intact hormone. It was also shown that the metabolic clearance rates of the subunits are very fast compared to those of the intact hormones, so that production rates of the subunits are faster than expected from the blood levels. Pituitary gland extracts show an α subunit pool which is larger than the β subunit pool, and variations in these proportions have been observed in pituitary tumors, some of which secrete a subunits preferentially. Factors which control subunit and intact hormone secretion are under investigation. (Weintraub, Rosen)

Inappropriate secretion of TSH has been shown in several patients with elevated serum thyroid hormone levels, sometimes in conjunction with peripheral resistance to thyroid hormone. One patient studied in detail revealed selective pituitary resistance to TSH, so that she was hyperthyroid in the face of elevated TSH production which was shown to be responsible for goiter formation. This is the first such case to be described. (Weintraub, Gershengorn)

C. Ectopic Protein Production by Tumors

Previous work on the secretion of placental proteins [chorionic gonadotropin (hCG), placental lactogen (hPL), placental alkaline phosphatase, and the hCGβ subunit] by lung tumors has been extended to a larger series of patients, and to subjects with other forms of cancer. Ectopic secretion of these proteins has now been found in malignant carcinoid, testicular carcinoma, and a few other sites. (Rosen, Weintraub)

Studies on in vitro production of placental proteins by two human cancer cell lines in culture have continued. One, a bronchogenic carcinoma, secretes mainly hCGα, while the other, a choriocarcinoma, secretes intact hCG. Several new, highly functional clones have been established, and functional transplants in athymic mice have been obtained. These cell lines have been compared with cultures of normal placental cells, which were found to secrete hCGα but not hCGβ or hPL and very little hCG. Studies on control of the synthesis of these proteins are in progress. (Rosen, Lieblich, Weintraub)

III. Adrenal Gland Secretion

The relation between ACTH receptors on the adrenal cortex cell and receptors for certain bacterial products which affect adrenal steroid secretion has been studied in steroid secreting cells in culture. Choleragen stimulates adenylate cyclase and steroid secretion in two adrenal and one Leydig cell line, one of which lacks the ACTH receptor. Therefore the cholera toxin receptor is not the same as the ACTH receptor. Furthermore, choleragen and ACTH do not interfere with each other in cells which retain ACTH receptors. In contrast, bacterial lipopolysaccharides (endotoxins) only stimulate the cell line which is rich in ACTH receptors, but endotoxins and ACTH also failed to show mutual interference. Since choleragen and endotoxins also fail to block each others action, all three substances appear to react with unrelated receptor sites on the adrenal cell. The bacterial products may directly activate steroid secretion in infections. (Wolff)

IV. Protein Structure

Studies on the structure of lipoproteins have been pursued to further understanding of the important interactions between proteins and lipid-containing structures such as biological membranes. A thermodynamic study of the self-association of the reduced alkylated apoprotein, apoA-II, has shown major changes in secondary and tertiary structure as a function of temperature, with maximum stability at 25°. The reaction is endothermic at lower temperature and exothermic at higher temperature, a phenomenon which may be important for protein structure in general. Interaction of apoA-II with various types of detergents has been compared with its interaction with lysolecithin, an ubiquitous lipid in biological systmes. Evaluation of the data obtained by circular dichroism, polarization and fluorescence is now in progress. (Osborne, Palumbo, Edelhoch).

Project No. Z01 AM 45000-08 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Thyroxine-Protein Interactions

Previous Serial Number: CEB 2c

Principal Investigators: S.-Y. Cheng, Ph.D., H. J. Cahnmann, Ph.D.,
M. Wilchek, Ph.D., R. N. Ferguson, Ph.D.,
D. Glinoer, M.D., M. Gershengorn, M.D.
J. Robbins, M.D.

Other Investigators: H. Edelhoch, Ph.D., E. Klima

Cooperating Units: None

Man Years:

Total: 2 3/4
Professional: 2 1/2
Other: 1/4

Project Description:

One purpose of this investigation is to study, on a molecular level, the interaction of thyroxine (T_4) with the binding sites on the principal T_4 carrier proteins in serum. The work done during the period covered by this report dealt with the localization of the binding sites for T_4 on prealbumin (PA). PA has two identical binding sites which show a slight degree of negative cooperativity ($n = 2$; $k = 5 \times 10^7 M^{-1}$, interaction factor $\alpha = .04$). The binding of T_4 is strongly inhibited after binding of 1.7 dansyl (DNS) residues per molecule of PA and dansylation is strongly inhibited after PA has been saturated with T_4. DNS-Gly also binds to PA ($n = 2$; $k = 3 \times 10^5 M^{-1}$, $\alpha = .80$), but can be displaced from the binding site by T_4. This shows that the binding sites for DNS and for T_4 are identical or close to each other. The site of the binding of DNS was therefore investigated.

PA was treated with [^3H]DNS-Cl. Acid hydrolysis of the labeled PA revealed Lys to be the only amino acid to which DNS had been covalently linked. Tryptic digestion of the labeled PA, after maleylation, followed by gel filtration, gave a labeled peptide which, after demaleylation, was further purified by cation exchange chromatography. Amino acid analysis of the labeled peptide showed that it consisted of residues 1 through 21 of PA which includes two Lys residues (9 and 15). It was shown by CNBr-cleavage of labeled PA at Met-13, followed by dialysis, that peptide 1 through 13 was not labeled. Removal of the N-terminal Val from the reamining labeled peptide by Edman degradation, followed by dansylation of the newly formed N-terminus (Lys-15), and acid hydrolysis yielded labeled $N^{\alpha,\varepsilon}$-di-DNS-Lys. Thus [^{3}H]DNS had attached itself to Lys-15 of PA which is located in the central channel formed by the four identical subunits of PA. Only two of the four Lys-15 residues of PA can react with DNS-Cl. Binding to the two other Lys-15 residues is apparently prevented by steric hindrance which can be deduced from the X-ray crystallographic data of Blake et al.

Affinity labeling of PA was also done with a derivative of T_4, viz. bromoacetyl-L-T_4 (BrAc-T_4). Two molecules of this ligand bind to PA with affinities of $\sim 10^8$ M^{-1} and $\sim 10^6$ M^{-1}, respectively, as determined by fluorescence titration of ANS-PA (see last year's report) with BrAc-T_4 at pH 7.4. These affinities are the same as those for T_4. Incubation of PA with [^{14}C]Br Ac-T_4 resulted in the covalent binding of the labeled ligand. The binding was inhibited in the presence of T_4 which suggests that T_4 and BrAc-T_4 bind to the same site. Amino acid analysis of an acid hydrolysate of the labeled PA showed the presence of labeled N^{ε}-carboxymethyl-Lys (derived from labeled BrAc-T_4-Lys by hydrolytic cleavage). Work to determine whether the point of attachment is, as in the case of DNS-Cl, Lys-15 is in progress.

The protein which transports about 3/4 of the serum thyroxine is the thyroxine-binding alpha globulin, TBG. This protein has been purified by affinity chromatography, employing T_4-Sepharose and has been used for the radioimmunoassay of TBG in human serum. The normal level was 1.4 ± .4 mg/dl. Parallel measurement of thyroxine-binding capacity showed a ratio of 13.9 μg T_4 per mg TBG. Since the molecular weight of TBG is 57,000 (measured by equilibrium sedimentation), this ratio indicates that TBG has a single binding site for T_4. Measurement of TBG in patients with hepatocellular carcinoma showed normal levels in many individuals but elevated levels in some. There was no correlation with α-fetoprotein levels. Similar procedures have been used for purification and measurement of TBG in rhesus monkeys, and are being applied to the study of TBG synthesis in monkey liver cells.

Keyword Descriptors: Prealbumin, Affinity labeling of prealbumin, Binding site for dansyl chloride on prealbumin, Binding site for bromoacetylthyroxine on prealbumin, Binding site for thyroxine of prealbumin, Competitive binding of dansylglycine and thyroxine on prealbumin.

Honors and Awards: None

Publications:

1. Ferguson, R. N., Edelhoch, H., Saroff, H. A. and Robbins, J.: Negative cooperativity in the binding of thyroxine to human serum prealbumin. Ferguson, R. N. and Cahnmann, H. J.: Addendum: Preparation of tritium-labeled 8-anilino-1-naphthalenesulfonic acid. Biochemistry 14: 282-289, 1975

Project No. Z01 AM 45001-03 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Analytical Procedures for Qualitative and Quantitative Determinations of Iodoamino Acid

Previous Serial Number: CEB 3c

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Keyword Descriptors: None

Honors and Awards: None

Publications: None

Project No. Z01 AM 45002-01 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Nonenzymic Model Reactions for the Conversion of 4-Hydroxyphenylpyruvic Acid to Homogentesic Acid

Previous Serial Number: None

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: T. Matsuura, Ph.D., I. Saito, Ph.D., M. Yamane, Ph.D., H. Shimazu, Ph.D.

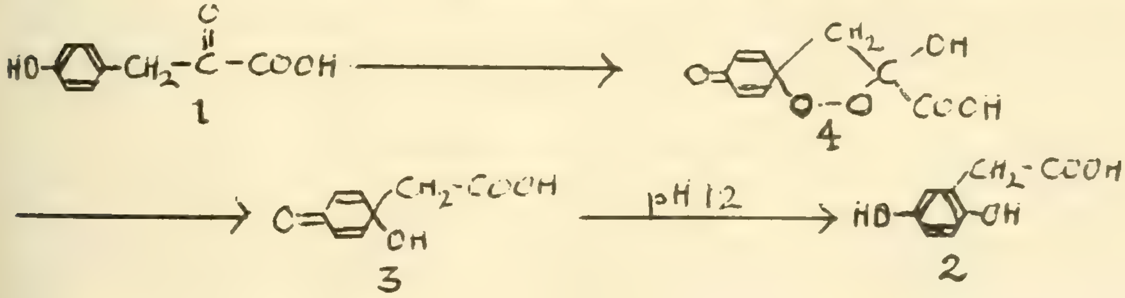
Cooperating Units: Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University, Kyoto, Japan

Man Years:

Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Previously reported work on this subject (see Project Report July 1, 1971 through June 30, 1972) has been resumed. Photooxygenation of 4-hydroxyphenylpyruvic acid (1) yields homogentesic acid (2). It has now been determined that the quinol 3 is an intermediate in this conversion. It is probably formed via the cyclic peroxide 4. Upon raising the pH, the intermediate 3 is converted to 2 by a mechanism analogous to that of the NIH shift. Thus, the mechanism of the conversion of 1 to 2 by photooxygenation is the same as that originally suggested by Goodman and Witkop for the biosynthesis of 2.



Keyword Descriptors: Photooxygenation of hydroxyphenylpyruvic acid, Nonenzymic model for the biosynthesis of homogentesic acid, Mechanism of the conversion of 4-hydroxyphenylpyruvic acid to homogentesic acid.

Honors and Awards: None

Publications:

1. Saito, I., Yamane, M., Shimazum, H., Matsuura, T. and Cahnmann, H. J.: Biogenetic type conversion of *p*-hydroxyphenylpyruvic acid into homogentesic acid. *Tetrahedron Letters* 9: 641-644, 1975.

Project No. Z01 AM 45003-01 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Model reactions for the synthesis of thyroxine residues in thyroglobulin

Previous Serial No.: None

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: J. Nunez, Ph.D., J. Pommier, Ph.D.

Cooperating Units: Unité de Recherche sur la Glande Thyroïde et la Régulation Hormonale, INSERM, Bicêtre, France

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

The purpose of this work is to investigate the mechanism by which two diiodotyrosine (DIT) residues in thyroglobulin (TG) couple to form a thyroxine (T_4) residue and, in particular, to determine the fate of the alanine side chain of DIT which is not incorporated into T_4 during this "coupling reaction". Various linear copolymers of Tyr with a single other amino acid were checked for their ability to form T_4 residues upon enzymic iodination. The rationale for this approach is that, if T_4 residues are formed in a simple copolymer consisting of only two amino acids, it should be easier to detect the "lost" alanine side chain than in TG which is composed of more than twenty different amino acids. It has been postulated by C. R. Harington that this alanine side chain is converted either to serine or to dihydroalanine. Although other possibilities are conceivable, our initial efforts were directed towards the detection of serine or dehydroalanine residues.

The search for a suitable copolymer led to the interesting finding that only one among several copolymers that were investigated yielded any detectable amounts of T_4 residues upon enzymic iodination. This copolymer consisting of Tyr and Lys, when treated with thyroid peroxidase in the presence of glucose, glucose oxidase, and iodide, gave a polymer containing monoiodotyrosine (MIT), DIT and T_4 residues. The latter were formed in 14-15% yield (based on the Tyr residues). Fractionation of the polymer on CM-cellulose, prior to iodination, gave a fraction which was capable of forming T_4 residues in even higher yields, indicating heterogeneity of the copolymer either in the degree of clustering of the Tyr residues or in preferred conformation in solution.

Only preliminary experiments were carried out to determine the presence or absence of Ser or dehydroalanine residues in the copolymers after the coupling reaction. Neither one of these residues was found, but more experiments using more refined techniques must be carried out before it can be stated that these residues are absent in the coupling product. It may be necessary to use in the future a copolymer containing [^{14}C]labeled Tyr. This would facilitate greatly the detection of [^{14}C]serine or of [^{14}C]alanine (after reduction of the dehydroalanine residues with sodium borohydride). So far only a few experiments were carried out with the unlabeled polymer. The detection of Ser was attempted by ion exchange resin and paper chromatography and that of dehydroalanine by treatment of the T_4-containing polymer with tritiated sodium borohydride, followed by paper chromatography.

An unsuccessful attempt was also made to couple the DIT residues in newly formed [^{14}C]labeled TG. Labeled neosynthesized TG was prepared by incubating sheep thyroid slices with [^{14}C]-tyrosine. The labeled TG, purified by gel filtration, was treated with the enzymic iodination mixture. [^{14}C]-MIT and [^{14}C]-DIT residues were formed, but no [^{14}C]-T_4 residues.

Keyword Descriptors: Models for the biosynthesis of thyroxine, Copolymers containing tyrosine as models for the biosynthesis of thyroglobulin, Coupling of diiodotyrosine residues in copolymers, Enzymic iodination of neosynthesized thyroglobulin.

Honors and Awards: None

Publications: None

Project No. Z01 AM 45004-04 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Structure of Polypeptide and Protein Hormones and Lipoproteins

Previous Serial Number: CEB 4c

Principal Investigators: H. Edelhoch, Ph.D., K. Ingham, Ph.D.,
G. Palumbo, Ph.D. and J. Osborne, Ph.D.

Other Investigators: R. E. Lippoldt, B. Brewer, M.D., and H. A. Saroff, Ph.D.

Cooperating Units: Molecular Disease Branch, NHLI and Laboratory of Biophysical Chemistry

Man Years:

Total: 5
Professional: 3 3/4
Other: 1 1/4

Project Description:

A thermodynamic study of the self-association of the reduced alkylated form apoA-II has been completed. The association constants have been obtained by measuring the concentration dependence of the mean residue ellipticity as a function of temperature. The variation of the free energy of association can be expressed as a function of temperature by:

$$\Delta F = A + BT + CT^2 + DT^3$$

The four coefficients are obtained by a least square fit of the data. From these coefficients the enthalpy, entropy and heat capacity change of the reaction can be obtained as a function of temperature. The association is extremely interesting since it involves major changes in second and tertiary structure and a maximum in stability is observed near 25°. This means that the reaction is endothermic at lower and exothermic at higher temperatures. The implications of these results for the understanding of the stability of all proteins is under study.

The interaction of apoA-II with cationic and anionic detergents has been studied by circular dichroism, polarization, and fluorescence in order to compare the behavior of these detergents with that of lysolecithin. The latter is a component of serum lipoproteins and most membranes. These studies are directed at unraveling the nature of the interactions between proteins and lipids so that the important biological structures containing lipoproteins may be better understood.

The motivation for pursuing the interaction of the hydrophobic fluorescence probe, 1,8-anilinonaphthalene sulfonate (ANS) with glycoprotein hormones was threefold. First, we were interested in the nature of the hydrophobic sites on the surface of these hormones. Second, we wanted to characterize the binding in order to extend the use of ANS as a probe for subunit interactions in other homologous hormones and in subunit hybrids. Finally, the binding of ANS appeared to be coupled to hormone self-association in such a way as to produce cooperativity in the binding curves. Although a number of authors have discussed various theoretical aspects of this phenomenon, there are few examples where the cooperative effects have been shown to derive exclusively from protein association. The interaction of ANS with human chorionic gonadotropin (hCG) and human luteinizing hormone (hLH) appeared to provide examples of both positive and negative cooperativity due to ligand-coupled self-association of the hormones.

Gel chromatographic and sedimentation equilibrium measurements indicate that both hormones undergo limited self-association to an extent which depends on the concentration of ANS. In the case of hLH, self-association proceeds only to the dimer, which has a single high affinity site ($Ka > 10^6$) for ANS. Binding to the monomer was sufficiently weak to be neglected. This leads to negative cooperativity in the binding of ANS to hLH. In the case of hCG, addition of ANS shifts the self-association first to the tetramer, which has 4 strong sites ($Ka \sim 10^{6.5}$) for ANS. At much higher ANS, the equilibrium shifts back to the dimer which has 4 sites of lower affinity. The ANS binding curves are biphasic and exhibit positive cooperativity. In both cases, the effects of hormone concentrations on both the position and shape of the ANS binding curve were quantitatively explained by the model. The ability of these hormones to alter their oligomeric state in response to a small ligand could be of some consequence for biological control. Efforts are in progress to find a physiological molecule which mimics the effects of ANS.

Keyword Descriptors: apoA-II, Lysolecithin, ANS, Gonadotropic hormone

Honors and Awards: None

Publications: None

1. Ferguson, R. N. and Cahnmann, H. J.: Negative cooperativity in the binding of thyroxine to human serum prealbumin. Biochemistry 14: 282-289, 1975.

2. Gwynne, J., Brewer, H. B., Jr. and Edelhoch, H.: The molecular behavior of ApoA-I in human high density lipoproteins. J. Biol. Chem. 250: 2269-2274, 1975.

3. Henkin, R. I., Lippoldt, R. E., Bilstad, J. and Edelhoch, H.: A zinc protein isolated from human parotid saliva. Proc. Nat. Acad. Sci. USA 72: 488-492, 1975.

Project No. Z01 AM 45005-12 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Measurement of Iodo Compounds in Biological Materials

Previous Serial Number: CEB 5c

Principal Investigator: C. G. Lewallen, M.D.

Other Investigators: H. Hoch, Ph.D.

Cooperating Units: Veterans Administration Center, Martinsburg, West Virginia

Man Years:

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

The consensus of current scientific opinion is that the principal biological result of thyroxine binding by plasma proteins is a reduction in free thyroxine (T_4) concentration, the proposed "active" form of the hormone involved in target cell penetration. The ultimate objective of our current research efforts is to ascertain whether or not any of the plasma proteins which bind T_4 play a positive, essential role in its transport from plasma to target cells. This is clearly not true of at least one of the strong protein binders of T_4, namely thyroxine binding globulin (TBG), since human subjects having congential absence of TBG are euthyroid. The situation with respect to the other binders is unknown. As a necessary first step, we are engaged in an attempt to identify as many as possible of the plasma protein binders to T_4 and triiodothyronine, both strong and weak. Results described here apply to T_4 only.

The methods we have used this year represent established and novel applications of zonal electrophoresis in agarose gel. Methods 1 and 4 below are from the literature. Methods 2 and 3 have been developed by us.

1. Conventional method - Plasma (or serum) mixed with radioactive T_4 serves as origin material and the stained pattern is compared with an autoradiograph. The underlying principle is that T_4 bound to a protein migrates not with its own mobility but with that of the T_4-protein complex. This method regularly demonstrates binding of T_4 by thyroxine binding prealbumin (TBPA), albumin, and TBG.

2. Post electrophoresis labeling - Immediately after electrophoresis of unlabeled serum, radioactive T_4 is applied to the wet gel in a manner comparable to that used for conventional protein stains. The gel is then dried, autoradiographed, and stained for protein in that order. The underlying principle is that in the wet gel a binding interaction between T_4 and a protein will produce a reduction in the diffusion of T_4 with the result that on the autoradiograph the protein zone will appear to be "stained" by radioactivity and visible above the background radioactivity. This method regularly demonstrates binding of T_4 by TBPA, albumin, α_1-lipoprotein, TBG, and β-lipoprotein.

3. Protein mobility method - Using special techniques, a gel is poured in such a fashion that although physically continuous it consists of 3 channels served by a common linear origin (multi-channel, common origin technique). T_4 is incorporated throughout the gel of the middle channel whereas the two lateral (control) channels have no additive. Following electrophoresis of serum the protein zones of the stained pattern are examined for bends caused by differences in electrophoretic mobility of a zone in the T_4-containing and control channels. Label is not required and measurements of migration distances are not necessary. The underlying principle is that binding of anionic T_4 will result in an increase in anodic mobility of the binding protein. Binding of T_4 by a process of ion exchange would not be expected to affect protein mobility. Other difficulties in interpretation are not considered here. This method suggests binding of T_4 by albumin, α_1-lipoprotein, β-lipoprotein, and two pre-β-lipoproteins.

4. Two dimensional cross electrophoresis - Unlabeled serum is electrophoresed in the first dimension. Linear origins of radioactive T_4 are then placed such that in the second dimension each separated protein component will either overtake, or be overtaken by, a migrating T_4 zone. The gel is then dried, autoradiographed, and stained for protein in that order. Linear zones of T_4 on the autoradiograph are examined for bends in the areas of contact with zones of binding proteins. The underlying principle is that binding of T_4 to a protein having a mobility significantly different from that of free T_4 may result in a detectable change in T_4 mobility. (This in contrast with method 3 in which binding is detected by a change in protein mobility.) Method 4 demonstrates binding of T_4 by TBPA, albumin, TBG, and β-lipoprotein.

Methods 2, 3 and 4 are all more sensitive than method 1 as indicated by the results. As our experience with these techniques has widened, it has become increasingly evident that the most serious limiting factor lies not with the methods per se but in the limited resolving power for protein components inherent in agarose gel electrophoresis. Accordingly, we are currently engaged in extending these studies using polyacrylamide gels and some of the techniques of immunoelectrophoresis.

Keyword Descriptors: Thyroxine binding, Plasma proteins, Thyroxine binding globulin, Radioactive T_4, Thyroxine binding prealbumin, albumin, α_1-lipoprotein, β-lipoprotein, Pre-β-lipoproteins, Cross electrophoresis

Honors and Awards: None

Publications: None

Project No. Z01 AM 45006-07 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Thyroid Iodoproteins

Previous Serial Number: CEB 6c

Principal Investigators: A. Haeberli, Ph.D., J. E. Rall, M.D., Ph.D., G. N. Salvatore, M.D.

Other Investigator: H. Edelhoch, Ph.D.

Cooperating Units: Office of the Director, NIAMDD

Man Years:

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

The goal of this project is to determine the number, size and chemical composition of the primary polypeptide chains of the major thyroid iodoprotein, 19 S thyroglobulin (MW 660,000.

In the presence of dissociating agents normally iodinated 19 S partially dissociates into a 12 S species (MW 330,000), the smallest noncovalently bound subunit. Following complete reduction and alkylation, hog, beef, rat and human thyroglobulin showed considerable size heterogeneity with still 12 S being the predominant band, when analyzed by SDS gel electrophoresis. In addition to the 12 S band there are approximately 10 bands with molecular weights between 10,000 and 300,000.

In contrast to the species mentioned above, guinea pig 19 S thyroglobulin, after reduction and alkylation, showed 3 major bands. Determination of the molecular weights of these proteins by several methods using SDS gel electrophoresis was unsatisfactory. The three proteins were isolated and examined by equilibrium centrifugation in 6 M guanidine HCl. By this method the molecular weights were as follows: A. 295,000, B. 210,000, C. 110,000. Amino acid analysis showed no major differences among those patients.

Thyroglobulin was isolated from normal guinea pig thyroids and from those of animals treated with thiouracil to produce thyroglobulin of a low iodine content. These preparations were fractionated by isopycnic centrifugation in RbCl into proteins of iodine content varying between 0.04% and 0.69%. When these fractions were reduced and analyzed by polyacrylamide gel electrophoresis in SDS, three species of proteins were observed. Species A with a molecular weight of 295,000, species B 210,000, and species C 100,000. Species A comprised 80% of the protein in thyroglobulin of 0.04% iodine and 13% in thyroglobulin of 0.69% iodine. Species C showed the opposite relationship comprising 10% of low iodine and 70% of high iodine thyroglobulin. Species B was relatively independent of the iodine content of thyroglobulin and comprised approximately 20% of the protein. Iodine analysis of the reported proteins showed species A to be lowest, species B intermediate and species C highest. It appears that the subunit composition of thyroglobulin is affected by the degree of iodination.

Keyword Descriptors: Thyroglobulin, Subunits

Honors and Awards: Honorary Doctorate of Medicine, Free University of Brussels, Brussels, Belgium - J. E. Rall

Publications:

1. Haeberli, A., Bilstad, J., Edelhoch, H. and Rall, J. E.: The elementary chain composition of guinea pig thyroglobulin. J. Biol. Chem., in press.

Project No. Z01 AM 45007-10 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Protein Synthesis in the Thyroid Gland

Previous Serial Number: CEB 7c

Principal Investigators: M. Izumi, M.D., J. Robbins, M.D., H. J. Cahnmann, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

Certain transplantable rat thyroid tumors do not contain thyroglobulin (TG) with a sedimentation coefficient of ∿19 S, but other soluble proteins which differ in shape and size from TG, but which share the property of forming immune complexes with anti-TG antibodies. A study of these abnormal TG's is of interest since it may throw light on the relationship between certain human thyroid cancers and the molecular properties of the thyroidal proteins.

We have undertaken work on the isolation and purification of the abnormal TG's found in Wollman's transplantable thyroid tumor 1-8R. Since these proteins could not be recovered from a $(NH_4)_2SO_4$ precipitate or be purified by DEAE-cellulose chromatography, we have used affinity chromatography for their isolation. The immunoglobulin fraction of rabbit anti-rat TG was covalently linked to agarose, either through an isourea bond (activation with CNBr) or through an ether bond (Affi-Gel 10). The latter type of bond has the advantage that it prevents hydrolytic leakage of antibody from the affinity column. All initial experiments were, however, carried out with CNBr-activated agarose (200 mg CNBr/ml). In a typical experiment the coupled agarose contained 9 mg immunoglobulin/ml and the binding capacity for rat TG was 1.4 mg/ml. Various solvents capable of eluting TG such as 3 M NaCNS or 0.05 M glycine NaOH, pH >11.5, caused partial breakdown of TG. Milder eluants such as 1 M guanidine or 2 M urea in 0.05 M glycine-NaOH, pH 10, eluted only 30-40% of bound TG whereas 0.05 M glycine - NaOH, pH 11 eluted 60%. Sucrose gradient centrifugation and polyacrylamide disc gel electrophoresis showed that exposure of TG to the latter solvent for 1 hr caused no breakdown. The amount of immunoprecipitable radioactivity (20-30%, double antibody assay) of a tumor extract obtained after *in vivo*-labeling with ^{125}I remained unchanged under these conditions. The pH 11 buffer was therefore used to elute TG-like tumor proteins. Exposure to pH 11 was limited to <20 min. Practically all immunoprecipitable material was retained on the affinity column at pH 7.1. The pH 11 eluate showed a radioactivity peak at ∿8 S in sucrose gradient centrifugation whereas the major peak of the original tumor extract was at ∿5 S. The 8 S peak had a wide base suggesting heterogeneity. Gel filtration on Bio-Gel A5m at pH 8.5 of either the pH 11 eluate or of the original tumor extract gave two radioactivity peaks, one of which (peak A) appeared in the void volume while the other one (peak B) was retarded to about the same extent as ovalbumin (MW ∿40,000). On a column of Bio-Gel A15m both A and B are retarded but not separated from each other. 6 M guanidine does not cause breakdown of peak A into smaller fragments. However, exposure of peak A to alkali (pH 11) for 1 hr causes the appearance of a radioactivity peak (on Bio-Gel A5m) which is more retarded than peak B. In spite of the apparently very high molecular weight of peak A indicated by its exclusion from Bio-Gel A5m, this peak sediments slowly (∿8 S) in the ultracentrifuge, suggesting that it has a low molecular weight or that it is a lipoprotein or that it has an unusually extended shape. These possibilities are now under investigation.

Keyword Descriptors: Abnormal thyroglobulin, Rat thyroid tumor, Affinity chromatography, Isolation and purification of thyroidal proteins, Immunoreactivity of abnormal thyroidal protein.

Honors and Awards: None

Publications:

1. Monaco, F., D'Armiento, M. and Robbins, J.: Incorporation of carbohydrates into abnormal thyroglobulin in an experimental rat thyroid tumor. *Endocrinol*. 94: 1445-1450, 1974.

2. Monaco, F., Salvatore, G. and Robbins, J.: The site of sialic acid into thyroglobulin in the thyroid gland. *J. Biol. Chem*. 250: 1595-1599, 1975.

Project No. Z01 AM 45008-02 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cyclic Nucleotide-dependent Protein Kinases of the Thyroid Gland

Previous Serial Number: CEB 14c

Principal Investigators: M. McClung, M.D., J. E. Rall, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

While TSH-dependent events in the thyroid gland are thought to be mediated by cAMP, the biochemical mechanisms by which these processes are stimulated are still unknown. Our project has involved the study of cyclic-nucleotide-dependent protein kinases of the thyroid gland. We improved our ability to purify cytoplasmic protein kinase so that its physical characteristics and its cAMP-binding and enzymatic activities can be better defined.

Of particular interest has been the study of the regulation of protein kinase activity. In bovine thyroid slices, we have demonstrated that TSH, by increasing intracellular concentrations of cAMP, does activate the protein kinase activity within the cell. We have been unable to demonstrate the presence of cytoplasmic protein modulators of kinase activity similar to that found in other tissues. We have also found that the cAMP receptor subunit of protein kinase is capable of being phosphorylated by the catalytic subunit of the intact molecule. We have also demonstrated this reaction using protein kinase purified from skeletal muscle. Further characterization of this autophosphorylation reaction is being made with the muscle enzyme because of the ease of its purification relative to thyroid protein kinase. Our experience thus far suggests that cAMP receptor phosphorylation may be an important regulator of the dissociative response of protein kinase to cyclic nucleotides.

We have also further characterized protein kinase activity in particulate fractions of bovine thyroid glands. cAMP-dependent protein kinase has been identified in both the microsomal and the nuclear fractions as well as the plasma-membrane-enriched fractions. The characteristics of this activity and the nature of the endogenous substrates of particulate protein kinases are being studied at this time.

Keyword Descriptors: Thyroid gland, Cyclic AMP, Protein phosphotransferase, Autophosphorylation

Honors and Awards: None

Publications: None

Project No. Z01 AM 45009-08 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies in Thyroid Disease

Previous Serial Number: CEB 8c

Principal Investigators: J. Robbins, M.D., B. Weintraub, M.D., M. Gershengorn, M.D.

Other Investigators: M. Izumi, M.D.

Cooperating Units: None

Man Years:

Total: 1 1/4
Professional: 1
Other: 1/4

Project Description:

Our current studies on human thyroid carcinoma are aimed at improving radioiodine therapy of this disease. The effectiveness of such therapy is limited by the rapid iodine release from the tumor after its incorporation into thyroglobulin. Until recently, the only agent known to block release of organic iodine from the thyroid gland was iodide ion. However, this agent is not used during ^{131}I therapy because it also would diminish tumor uptake. In previous studies we showed that lithium ion blocks the release of iodine from the normal thyroid gland and is effective in the therapy of hyperthyroidism for the same reason. We therefore investigated the blocking effect of Li^+ in a patient with highly functional thyroid carcinoma. Li^+ decreased the disappearance rate of a tracer dose of ^{131}I from the whole body and from a tumor mass from control values of 0.126 and 0.122/day to .09 and .049/day, respectively. Although this was expected to increase the therapeutic:toxic ratio of ^{131}I, an unanticipated increase in blood ^{131}I followed a subsequent therapy dose, presumably due to increased tumor radiation, and resulted in significant bone marrow depression. Thus, Li^+ is effective as an adjunct in ^{131}I therapy but must be used with caution.

The demonstrated blocking effect of Li^+ will also be employed as an adjunct to the use of TRH in enhancing tumor uptake prior to ^{131}I therapy. Currently, bovine TSH or induction of hypothyroidism are the major means to stimulate ^{131}I uptake. The former can produce blocking antibodies and the latter produces significant patient discomfort. TRH would cause release of endogenous TSH while the patient is still euthyroid, and Li^+ would prevent feedback inhibition by released thyroid hormone. Our initial studies were carried out in a series of athyreotic patients with poorly functional thyroid carcinoma. Oral TRH, 100 mg q 12 hr, administered for 14 days, produced a persistent rise in serum TSH levels, as well as enhanced ^{131}I uptake by thyroid tissue. In future studies on highly functional carcinoma, the combined effects of TRH and Li^+ will be investigated.

Studies have also been initiated to evaluate the usefulness of serum thyroglobulin assay in the diagnosis of thyroid tumors. Van Herle has claimed that the very high levels seen in thyroid carcinoma may be diagnostic of this disease. A radioimmunoassay for human and rat thyroglobulin has been developed. The former was used to study sera obtained in the Marshall Islands from a population exposed to radioiodine fallout radiation as a possible means to detect thyroid abnormality. In this population, however, most of the young Rongelap persons, in whom a very high incidence of thyroid nodules and carcinoma had been produced by the radiation, had had prior thyroid surgery. Compared to control series from the island groups and from the United States, the people with a history of thyroid disease had a significantly lower serum thyroglobulin level. This was apparently due to radiation destruction of the remaining thyroid tissue and also to the use of suppressive thyroxine therapy. The Uterik people, exposed to a lower dose of radiation and not on thyroid suppression therapy, had normal serum thyroglobulin levels.

Keyword Descriptors: Thyroid diseases, Thyroid carcinoma, Radioiodine, Thyrotropin releasing hormone, Lithium effects, Thyroglobulin radioimmunoassay.

Honors and Awards: None

Publications:

1. Robbins, J., Rall, J. E. and Gorden, P.: The thyroid and iodine metabolism. In Bondy, P. and Rosenberg, L. (Eds.): Duncan's Diseases of Metabolism. Philadelphia, Saunders, 1974, pp. 1009-1104.

2. Robbins, J. Thyroid: its functions, hormones and diseases. In Osol, A. and Pratt, R. (Eds.): U.S. Dispensatory. Philadelphia, J. B. Lippincott, 1973, pp. 1190-1197.

3. Carlson, H. E. and Robbins, J.: Effects of hormones and nucleotides on ciliary beating in frog esophagus and guinea pig trachea. Life Sciences 14: 2413-2426, 1974.

Project No. Z01 AM 45010-05 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Ectopic Placental Protein and Subunit Production by Tumors

Previous Serial Number: CEB 9c

Principal Investigators: S. W. Rosen, Ph.D., M.D., B. D. Weintraub, M.D.

Other Investigators: L. Broder, M.D., H. Hansen, M.D., F. Muggia, M.D., H. Sussman, M.D., J. Cochran, M.D., P. Scardino, M.D., T. Waldmann, M.D., R. Kahn, M.D., P. Gorden, M.D., H. Brereton, M.D., L. Panton, M.D., I. Stotler, B.S., B. Stannard, B.S., Y. Kadesky, B.S., J. Lieblich, M.D., A. Rabson, M.D., C. Kim, M.D., P. Kohler, M.D., W. Sindelar, M.D., J. Chou, Ph.D., J. Robinson, M.D., E. Maxwell, Ph.D., G. Cox, Ph.D.

Cooperating Units: NCI Oncology Unit, VA Hospital, Washington, D.C.; Pathology Dept., Stanford University Medical Center, Palo Alto, CA; Dept. of Urology, University of Texas, Dallas, TX; Surgery Branch, NCI; Metabolism Branch, NCI; Diabetes Branch, NIAMDD; Radiation Branch, NCI; Division of Cancer Biology and Diagnosis, NCI; Endocrine Division, Baylor College of Medicine, Houston, TX; Laboratory of of Biomedical Sciences, NICHD; Laboratory of Molecular Biology, NIAMDD.

Man Years:

Total: 3 1/4
Professional: 1 3/4
Other: 1 1/2

Project Description:

A) In Vivo Studies

Prospective analysis of sera from patients at the Washington, D.C. VA Hospital with inoperable lung cancer continues. More than 200 such sera have been examined for ectopic production of the placental proteins (chorionic gonadotropin (hCG), placental lactogen (hPL), placental alkaline phosphatase (PAP), and the beta subunit of hCG (hCG-β). At least* 12 positive cases have been detected thus far for an overall incidence of @ 6%. Neoplastic production of these cancer "markers" appears to be discordant. Thus, serum from 5 of the new cases had hCG only, 3 had hPL and 3 had PAP. Only one case had more than one "marker" in the serum. (Broder, Hansen, Muggia, Stotler, Stannard).

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*screening is not yet complete in a few cases.

A similar prospective analysis has begun of sera from patients with oat cell carcinoma of the bronchus, admitted to the Clinical Center for radiotherapy (Brereton, Stannard, Stotler). Patients with ectopic placental protein or subunit production detected in this screening will have extensive endocrine evaluation.

Retrospective analysis of sera from patients with malignant carcinoid tumors has detected 4/25 with ectopic production of the alpha subunit of the glycoprotein tropic hormones (α). These data argue against a theory of Levine and Metz, who postulate that ectopic hormone production by carcinoids is limited to a select group of polypeptides, not including α (Stannard, Stotler).

Retrospective anlaysis has begun of sera from patients with other carcinomata. Sera from cases of testis, prostate, pancretic islet and pancreatic adenocarcinoma are being screened for placental protein and subunit production. Partial data from 62 cases of testis cancer reveal 11 with production only of hCG or hCG-β, 4 with both hCG (or hCG-β) and hPL circulating, but none with hPL alone -- although one of the 4 with two placental proteins in the serum had higher levels of hPL than of the hCG marker (Cochran, Scardino, Waldmann). One case has been detected of hCG production by a carcinoma of the prostate (Broder). Preliminary analysis of sera from patients with islet cell adenomas and carcinomas has also revealed several instances of hCG and α production (Kahn, Gorden).

Four men with metastatic carcinoma (2 - bronchus, 1 - stomach, 1 - primary unknown) and ectopic placental protein production (1 -hCG and α, 1 - hPL, 1 - hCG, 1 hCG and hPL) were studied with serial retrospective measurements of the ectopic protein concentrations in serum, correlated with objective assessment of clinical status. The biochemical marker and clinical response were documented during treatment with a variety of agents and during periods without therapy. Of 9 assessable periods, 7 showed concordance of biochemical marker and clinical response (Muggia, Hansen).

Sera from a woman in her 60's and a man in his 40's, neither of whom were known to be harboring a neoplasm, nontheless contained hCG in concentrations exceeding 1 ng/ml. The presence of hCG was confirmed in a radioimmunoassay in which the antiserum displayed apparent cooperativity with hCG but not with any other ligands tested. Additional experiments are planned to characterize this circulating hCG further and to search for occult neoplasm. These data, if sustained, suggest that the "ectopic" production of placental proteins by certain neoplasms is one end of a continuous quantitative spectrum rather than a "qualitative" marker for cancer.

B) In Vitro Studies

We have compared rates of secretion of hCG, α, and hCG-β by clonal cell strains derived from established *in vitro* cultures of a bronchogenic carcinoma (ChaGo) and a choriocarcinoma (JEG). Clones showing the highest secretion rates of either hCG or subunits were studied: ChaGo-K1, a new clonal strain, and JEG-3, a previously reported clonal strain. Cells were grown under identical conditions in the same laboratory. ChaGo-K1 secreted only α whereas JEG-3 secreted primarily hCG: average peak secretion rates in picomoles/day/mg protein for ChaGo-K1 were hCG < 0.1, α 160-190, and hCG-β < 0.1, and for JEG-3 were hCG 8-18, α < 1.0, and hCG-β < 1.0. The ChaGo-K1 secretion rate of α was several hundred fold greater than any of our previously reported ChaGo clones, all of which had secreted small amounts of biologically active hCG. Significant quantities of estradiol and progeserone accumulated in the media of both clonal strains; in contrast, JEG-3 cells secreted significant quantities of hPL while ChaGo-K1 secreted undetectable amounts. Thus, under identical conditions, a clonal cell strain derived from a bronchogenic carcinoma secreted only α subunit whereas a clonal strain derived from a choriocarcinoma secreted primarily hCG -- implying major differences in cellular control mechanisms. Moreover, the ectopic secretion of α exceeded the trophoblastic secretion of hCG, suggesting that in certain cases ectopic protein production may be even more efficient than nonectopic production (Lieblich, Rabson, Kim, Kohler).

We have begun to study the effects of various chemotherapeutic agents on the *in vitro* production of α by ChaGo cells. Preliminary data show markedly disparate effects on α production by different agents. There appears to be good correlation between the effect of an agent *in vitro* and the patient's *in vivo* response to the same agent (Broder - Z01CM0630906M0).

ChaGo cells inoculated into a strain of "nude" (athymic) mice formed tumors at the sites of injection. These tumors, like the cells from which they derived, appear to be functioning and making α subunit (Sindelar, Rabson).

New placental cell lines and a clonal strain have been established in culture from a normal human placenta at term. These cells secrete α but not hCG-β or hPL. Complete hCG was detected but its secretion was less than 1% of α. The placental lines also produce an intracellular alkaline phosphatase similar to the placental isozyme. Availability of these functioning cell lines derived from normal placenta will allow comparison of placental protein biosynthetic and regulatory mechanisms with our neoplastic cell lines (Chou, Robinson, Sussman).

A poly (A) containing mRNA fraction isolated from normal human term placentas catalyzes the biosynthesis of a material behaving immunochemically like hPL but migrating on SDS-PAGE as a species of apparently higher molecular weight. The question of a possible precusor product relationship with hPL is being studied (Cox, Maxwell).

We have already reported differences in physicochemical properties between ectopic α purified from tumor and standard α purified from placenta. Heterogeneity of hPL and hCG have also been observed in gel chromatography of serum from patients with ectopic production. For hCG, heterogeneity was confirmed in ion-exchange chromatography (Panton, Kadesky). The relationship of the heterogeneous forms of circulating hPL to the product of apparent larger molecular size noted in vitro (see above) is still unclear.

Keyword Descriptors: Placental proteins, Ectopic hormone production, Chorionic gonadotropin, Alpha subunit, Placental lactogen, Bronchogenic carcinoma, in vitro, Choriocarcinoma, in vitro, Placental cells in vitro, Cancer markers

Honors and Awards: None

Publications:

1. Rosen, S. W. and Weintraub, B. D.: Ectopic production of the isolated alpha subunit of the glycoprotein tropic hormones: a quantitative marker in certain cases of cancer. New Engl. J. Med. 290: 1441-1447, 1974.

2. Thomas, D. W., Rosen, S. W., Kahn, R. C., Temple, R. and Papadopoulos, N. M.: Macromolecular lactic acid dehydrogenase: a cause of increased serum lactate dehydrogenase activity. Ann. Intern. Med. 81: 434-438, 1974.

3. Rosen, S. W., Weintraub, B. D., Vaitukaitis, J. L., Sussman, H. H., Hershman, J. M. and Muggia, F. M.: Placental proteins and their subunits as tumor markers. Ann. Intern. Med. 82: 71-83, 1975.

4. Rosen, S. W. and Weintraub, B. D.: Humours, tumors and caveats. Ann. Intern. Med. 82: 274-276, 1975.

Project No. Z01 AM 45011-03 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies with Luteinizing Hormone-Releasing Factor (LRF)

Previous Serial Number: CEB 11c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: A. D. Rogol, M.D., J. Rochefort, Ph.D.

Cooperating Units: Ayerst Laboratories, New York, New York

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Keyword Descriptors: None

Honors and Awards: None

Publications: None

Project No. Z01 AM 45012-02 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Growth Hormone Heterogeneity

Previous Serial Number: CEB 12c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: R. C. Dimond, M.D.

Cooperating Units: Walter Reed Army Institute of Research

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Ten sera from 4 patients with acromegaly, serum from a normal individual, and purified pituitary growth hormone (hGH) were examined by gel chromatography on Sephadex G-100. The data suggest that there may be as many as four circulating components of immunoreactive hGH, two of which elute before albumin. At least one of the GH components (peak III, K_{av} 0.4, app. mol wt @ 20,000) appears to be secreted both in response to TRH in acromegalics and in response to insulin-induced hypoglycemia in normal individuals.

Keyword Descriptors: Growth hormone heterogeneity, Acromegaly

Honors and Awards: None

Publications:

1. Dimond, R. C., Wartofsky, L. and Rosen, S. W.: Heterogeneity of circulating growth hormone in acromegaly. J. Clin. Endocrinol. Metab. 39: 1133-1137, 1974.

Project No. Z01 AM 45013-03 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: TSH and Prolactin Studies; Subunits of Glycoprotein Hormones

Previous Serial Number: CEB 13c

Principal Investigators: B. D. Weintraub, M.D., S. W. Rosen, Ph.D., M.D.

Other Investigators: M. Gershengorn, M.D., I. Kourides, M.D., F. Maloof, M.D., E. C. Ridgway, M.D., R. Re, M.D., A. Rogol, M.D., R. Dimond, M.D.

Cooperating Units: Harvard Medical School, Boston, Massachusetts; Walter Reed Army Institute of Research, Washington, D.C.

Man Years:

Total: 2 1/4
Professional: 1 1/4
Other: 1

Project Description:

A) Positive Cooperativity in Radioimmunoassay

Previous work from our laboratory has shown that certain antisera generated against polypeptide hormones and their subunits may show positive cooperativity in radioimmunoassay. We have continued to develop and apply such "cooperative" assays and have compared them to conventional assays in sensitivity and specificity. Recent data suggest that the "cooperative" assays may be particularly useful in the measurement of complete glycoprotein hormones and in distinguishing them from their free subunits.

B) Measurement of Glycoprotein Hormone Subunits in Serum and Pituitary Glands

We have developed a variety of antisera specific for the common alpha subunit of the glycoprotein hormones as well as to the specific beta subunits of these hormones. Such antisera have permitted examination of the regulation of subunit and complete glycoprotein trophic hormone biosynthesis in response to hypothalamic releasing factors and to target organ hormones. These studies have shown that after an intravenous bolus of thyrotropin releasing hormone (TRH) free subunits of TSH appear in the human circulation prior to TSH. Moreover, labeled TSH is not dissociated into subunits when infused into these same patients. These data suggest that subunits are actually secreted by the pituitary gland in response to TRH.

Recent studies have demonstrated that luteinizing hormone releasing-hormone (LHRH) also causes the appearance of free subunits in the human circulation. Currently we are examining the effect of thyroid hormones and gonadal steroids on the subunit responses to both TRH and LHRH.

Extracts of normal human pituitary glands show a large pool of free alpha subunits relative to the specific beta subunits of TSH and gonadotropins. Extracts from pituitary tumors show altered quantitative and qualitative subunit concentrations. In certain cases such tumors are also associated with excessive secretion of free alpha subunits.

Work is in progress to develop radioimmunoassays for rat and other animal glycoprotein hormone subunits. Using these assays we are developing methods for studying pituitary biosynthesis, secretion and combination of subunits both in vivo and in vitro.

C) Metabolic Clearance and Production Rates of Various Alpha and Beta Subunits

Using the constant infusion to equilibrium technique previously developed for TSH, we have measured the metabolic clearance and production rate of various subunits in man. All subunits are cleared much more rapidly from the circulation than complete glycoprotein hormones, accounting for their low serum concentrations even in the presence of a high production rate. In fact, the production rate of the free common alpha subunit is actually higher than that of any glycoprotein hormone.

D) Inappropriate Secretion of TSH

We have now discovered several cases of inappropriately high TSH secretion in the presence of elevated serum concentrations of total and free thyroid hormones. Certain of these cases show generalized target organ resistance to the action of thyroid hormone. Recently we have studied a patient with selective pituitary resistance to the action of thyroid hormone. In this case the resistance was partial, and we are currently studying the effects of long-term treatment with large doses of T_3.

E) Human Prolactin Studies

Studies continue on the relationship of prolactin, TSH, and subunit secretion in response to TRH, suckling, thyroid hormones, and glucocorticoids. Whereas TRH causes release of both TSH, subunits and prolactin, suckling only causes the release of prolactin. Thyroid hormones totally block basal and TRH-stimulated TSH secretion but only partially block prolactin secretion; glucocorticoids partially block basal and TRH-stimulated TSH secretion but have no effect on prolactin secretion. The relationship of the various forms of circulating TSH and prolactin is also being studied.

Keyword Descriptors: Thyrotropin, Prolactin, Subunits of glycoprotein hormones, Thyrotropin releasing hormone, Positive cooperativity, Radioimmunoassay, Pituitary glands, Biosynthesis of hormones, Metabolic clearance and production rates, Target organ resistance

Honors and Awards: None

Publications:

1. Kieffer, J. D., Weintraub, B. D., Baigelman, W., Leeman, S. and Maloof, F.: Homologous radioimmunoassay of thyrotrophin in rat plasma. Acta Endocrinologia 76: 495-505, 1974.

2. Gautvik, K. M., Tashjian, A. H., Jr., Kourides, I. A., Weintraub, B. D., Graeber, C. T., Maloof, F., Suzuki, K. and Zuckerman, J. E.: Thyrotropin-releasing hormone is not the sole physiologic mediator of prolactin release during suckling. New Eng. J. Med. 290: 1162-1165, 1974.

3. Ridgway, E. C., Singer, F. R., Weintraub, B. D., Lorenz, L. and Maloof, F.: Metabolism of human thyrotropin in the dog. Endocrinology 95: 1181-1185, 1974.

4. Rogol, A. D. and Rosen, S. W.: Alteration of human and bovine prolactins by a chloramine T radioiodination: comparison with lactoperoxidase-iodinated prolactins. J. Clin. Endocrinol. Metab. 39: 379-382, 1974.

5. Dimond, R. C. and Rosen, S. W.: Chromatographic differences between circulating and pituitary thyrotropins. J. Clin. Endocrinol. Metab. 39: 316-325, 1974.

6. Kourides, I. A., Weintraub, B. D., Ridgway, E. C. and Maloof, F.: Pituitary secretion of free alpha and beta subunit of human thyrotropin in patients with thyroid disorders. J. Clin. Endocrinol. Metab. 40: 872-885, 1975.

Project No. Z01 AM 45014-04 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Adrenal Secretion

Previous Serial Number: CEB 15c

Principal Investigators: J. Wolff, M.D., Ph.D.

Other Investigators: G. H. Cook

Cooperating Units: None

Man Years:

Total: 3/4
Professional: 1/4
Other: 1/2

Project Description:

Studies on adrenal secretion have been extended to the effects of choleragen and bacterial endotoxins on steroid secreting cells in culture. The aim has been to investigate the relation between the ACTH receptor and those for bacterial products which may or may not be activated directly in certain infections.

Choleragen stimulates steroid secretion and adenylate cyclase in three cell lines: two adrenal tumor lines (Y-1 and OS-3), the latter having no functional ACTH receptor, and a receptor-deficient Leydig cell line (I-10). Sensitivity for half-maximal stimulation varies from 3 to 36 pM, the I-10 line being the most sensitive. Latency before the onset of steroidogenesis is longer in OS-3 and I-10 cells than in the Y-1 line.

Evidence is presented that the ACTH and choleragen receptors are different: 1) choleragen does not, during its latency, block the response to ACTH; 2) choleragen stimulates steroidogenesis in the absence of a functioning ACTH receptor (OS-3); 3) ACTH does not block the choleragen effect in this cell.

In contrast, bacterial lipopolysaccharides (endotoxins) stimulate steroidogenesis only in the Y-1 cell and not in the OS-3 or I-10 lines. Although both hormone and lipopolysaccharide responses are lost in these lines, there was no interaction between these sites as judged by the failure of lipopolysaccharides to block, during their latency, the response to ACTH in line Y-1. Moreover, the choleragen and lipopolysaccharide site also appear to be distinct since the response to choleragen is not altered in the presence of lipopolysaccharides.

Keyword Descriptors: Adrenal tumor cells, Leydig tumor cells, Choleragen (enterotoxin), Lipopolysaccharides (endotoxin), Steroid secretion, Adenylate cyclase, Membrane receptors

Honors and Awards: None

Publications: None

Project No. Z01 AM 45015-05 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Thyroid Plasma Membranes

Previous Serial Number: CEB 16c

Principal Investigators: J. Wolff, M.D., Ph.D., E. L. Hewlett, M.D.

Other Investigators: G. H. Cook

Cooperating Units: Section on Membrane Regulation - C. Londos and M. Rodbell
Section on Biochemistry of Cell Regulation - L. Kohn

Man Years:

Total: 2
Professional: 1 1/4
Other: 3/4

Project Description:

TSH binding has been studied in beef thyroid membranes. It was concluded that (1) TSH binding is relatively stable and requires major structural alterations to affect binding; (2) the effect of activators and inhibitors on TSH binding is independent of TSH concentration and medium conditions; (3) most modulators act in the cyclase system at a stage beyond hormone binding; (4) the response of TSH binding to nucleotide triphosphates indicates that the thyroid membrane system is different from other membrane systems; and (5) the relationship between hormone binding and cyclase activation is complex and direct correlation between binding and activation is not presently possible. A search for higher affinity binding sites is under way.

We have also investigated the role of the TSH subunits in the binding and activation processes of various thyroid tissues. The binding of bovine TSH (thyroid stimulating hormone), LH (luteinizing hormone), and their subunits to the TSH receptor of beef thyroid membranes was compared to stimulation by these agents of adenylate cyclase, glucose oxidation in dog thyroid slices, and the secretory process in mouse thyroids *in vitro* (colloid droplet formation) and *in vivo* (hormone release). The β-subunits of TSH and LH can bind to the TSH receptor and can activate thyroid function *in vitro*. In contrast, the α-subunit of TSH binds negligibly to the TSH receptor and has very low potency for stimulation of thyroid function (except for colloid droplet formation). Neither binding nor the biological activity of the β-subunits can be accounted for by TSH contamination, whereas this cannot be ruled out for α-TSH. LH binds to the TSH receptor even better than the β-subunit of TSH but the increased binding does not result in a corresponding activation of thyroid function. Neither α- nor β-TSH alone can induce more than 4-8% of the response to intact TSH in any of the investigated parameters. It is proposed that the β-subunit has within its structure the primary determinants which are necessary to stimulate biological activity, whereas the α-subunit imposes conformational changes on the β-subunit which in intact TSH promote binding and biological activity commensurately but in LH promote only binding.

In addition the role of nucleotides in adenylate cyclase activation has been studied in a variety of tissues. 5'-Guanylylimidodiphosphate (Gpp(NH)-p) stimulates adenylate cyclase activity in plasma membranes isolated from frog and salmon erythrocytes, from rat adrenal, hepatic, and fat cells, and from bovine thyroid cells. The nucleotide acts cooperatively with the various hormones (glucagon, secretin, ACTH, thyrotropin, and catecholamines) that stimulate these adenylate cyclase systems with resultant activities that equal or exceed those obtained with hormone plus GTP or with fluoride ion. In the absence of hormones, Gpp(NH)p is a considerably more effective activator than GTP, and, under certain conditions of incubation, stimulates rat fat cell adenylate cyclase to about 20 nmoles of 3',5'-adenosine monophosphate mg protein per min. The nucleotide activates frog erythrocyte adenylate cyclase when the catecholamine receptor is blocked by the competitive antagonist, propranolol, and activates the enzyme from an adrenal tumor cell line which lacks functional ACTH receptors. In contrast, Gpp(NH)p does not stimulate adenylate cyclase in extracts from _Escherichia coli_ B. Gpp(NH)p appears to be a useful probe for investigating the mechanism of hormone and nucleotide action on adenylate cyclase systems in eukaryotic cells.

Finally, a soluble adenylate cyclase is being purified from _Bordetella pertussis_, in part, to serve as a model for the properties of other cyclases, particularly that from the thyroid. The work underway at present consists of evaluation of the control mechanisms which are regulatory for toxic product release from log growing _Bordetella pertussis_ organisms. Several enzyme systems are being studied with reference to their role in this release process. Eventual objective is to isolate and characterize the toxic products of bacterial origin and investigate their mechanism of influencing metabolism of mammalian cells.

Keyword Descriptors: TSH receptor, TSH subunits, Thyroid membranes, Adenylate cyclase, Glucose oxidation, Colloid droplets, Guanylylimidodiphosphate, _B. pertussis_

Honors and Awards: None

Publications:

1. Moore, M. W. and Wolff, J.: Thyroid-stimulating hormone binding to beef thyroid membranes. _J. Biol. Chem._ 249: 6255-6263, 1974.

2. Wolff, J., Winand, R. J. and Kohn, L. D.: The contribution of subunits of thyroid stimulating hormone to the binding and biological activity of thyrotropin. Proc. Nat. Acad. Sci. USA 71: 3460-3464, 1974.

3. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J. and Rodbell, M.: 5'-Guanylylimidodiphosphate, a potent activator of adenylate cyclase systems in eukaryotic cells. Proc. Nat. Acad. Sci. USA 71: 3087-3090, 1974.

Project No. Z01 AM 45016-05 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Thyroid Hormone Secretion

Previous Serial Number: CEB 17c

Principal Investigators: J. Wolff, M.D., Ph.D., B. Bhattacharyya, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

Studies on the role of microtubules or tubulin in secretory processes have led us into two areas of investigation: 1) a detailed study of ligand-tubulin interactions with colchicine and its analogues, particularly those of interest clinically such as trimethylcolchicinic acid, vinblastine, and podophyllotoxin, and ANS; and 2) the possible existence and nature of membrane-bound nonmicrotubule tubulin.

Colchicine, which does not fluoresce in aqueous media and organic solvents, exhibits marked fluorescence on combination with brain tubulin, with a corrected excitation maximum at 362 nm, an emission maximum at 435 nm, and a quantum yield of 0.03. From fluorescence measurements it was found that rat brain tubulin binds 0.83 moles of colchicine per dimer (molecular weight 110,000) with an association constant of 3.2 μM^{-1} at pH 7.0 and 37°. These results are in excellent agreement with those obtained with the binding of [^{3}H]-colchicine. The enthalpy of binding is 10 kcal/mole, with an entropy change of 62 entropy units. The fluorescence can be ascribed to the tropolone moiety. However, the A ring of colchicine is also involved in binding. Denaturing agents abolish fluorescence, whereas podophyllotoxin, another antimitotic agent, decreases fluorescence competitively. Fluorescence is a convenient method for determining the binding of colchicine to tubulin that does not require the separation of free colchicine from bound colchicine and yields values for physical and biochemical parameters that are in excellent agreement with those obtained from the binding of [^{3}H]-colchicine.

Rat brain tubulin binds 1 mole of 1-anilino-8-naphthalene sulfonate ANS per dimer (110,000 daltons) with an association constant of 3.2×10^5 M^{-1}. The quantum yield of ANS fluorescence is increased 120-fold over that in water to $\phi = 0.48$ and there is a hypsochromic shift of 56 nm to an emission maximum of 460 nm. There is energy transfer from tryptophan to bound ANS. Vinblastine and Ca^{2+} enhance ANS fluorescence in tubulin by 35%-40%; this can be ascribed to an increased quantum yield, rather than changes in the affinity constant or when colchicine binding has decreased to 50%. It is concluded that the colchicine- and ANS-binding sites occupy different regions of the tubulin molecule.

Brain and thyroid tissue contain membrane-bound colchicine-binding activity that is not due to contamination by loosely-bound cytoplasmic tubulin. This activity can be solubilized to the extent of 80-90% by treatment with 0.2% Nonidet P40 with retention of colchicine binding. Extracts so obtained contain a prominent protein band in disc gel electrophoresis that with tubulin. Membranes, and the solubilized protein therefrom, exhibit ligand binding properties like tubulin; for colchicine the K_A $\sim 1 \times 10^6$ M^{-1} in brain and $\sim 0.6 \times 10^6$ M^{-1} in thyroid; for vinblastine the K_A is $\sim 8 \times 10^6$ M^{-1} for both tissues; and for podophyllotoxin the Ki is $\sim 2 \times 10^{-6}$ M for both tissues. Displacement by analogues of colchicine is of the same order as for soluble tubulin. Although membrane-bound colchicine-binding activity shows greater thermal stability and a higher optimum binding temperature (54° *vs.* 37°C) than soluble tubulin, this appears to be the result of the membrane environment since the solubilized binding activity behaves like the soluble tubulin. Antibody against soluble brain tubulin reacts with membranes and solubilized colchicine-binding activity from both brain and thyroid gland. We conclude that brain and thyroid membrane preparations contain firmly-bound tubulin or a very similar protein.

Keyword Descriptors: Tubulin, Fluorescence, Colchicine, ANS, Vinblastine, Calcium, Quantum yield, Brain membranes, Thyroid membranes

Honors and Awards: None

Publications:

1. Bhattacharyya, B. and Wolff, J.: Promotion of fluorescence upon binding of colchicine to tubulin. *Proc. Nat. Acad. Sci. USA* 71: 2627-2631, 1974.

2. Bhattacharyya, B. and Wolff, J.: The interaction of 1-anilino-8-naphthalene sulfonate with tubulin: a site independent of the colchicine-binding site. *Arch. Biochem. Biophys*. 167: 264-269, 1975.

Project No. Z01 AM 45017-01 CEB
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: The Mechanism of Action of Lithium

Previous Serial Number: None

Principal Investigators: J. Wolff, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

The mechanism of action of Li^+ in manic depressive psychosis is not understood. Because side reactions to this cation are easier to study we have used adrenal secretory responses to investigate Li^+ effects. Two mM LiCl causes significant inhibition by ACTH-stimulated steroid secretion. With preincubation the required concentration can be reduced to 1 mM. Using colchicine, preliminary findings suggest that Li^+ may act by stabilizing microtubules. Direct studies of this are under way.

Keyword Descriptors: Lithium, Adrenal secretion

Honors and Awards: None

Publications:

1. Berens, S. C. and Wolff, J.: The Endocrine Effects of Lithium. In Johnson, F. N. (Ed.): Lithium Research and Therapy. London, Academic Press, 1975, pp. 443-472.

DIABETES BRANCH ANNUAL REPORT
SUMMARY
1974 - 1975

The Diabetes Branch focuses on the interaction of peptide hormones with their specific receptors on target cells with particular emphasis to insulin, growth hormone and NSILA-s, a well characterized circulating insulin-like peptide. Further, we apply these studies extensively to disease states in man.

In our studies of the general mechanism by which target cells regulate their sensitivity to hormone, we have uncovered two previously undescribed mechanisms which appear to apply widely to polypeptide hormones as well as to other hormone systems. The first is negative cooperativity. The affinity of target cell receptors for hormone appears not to be fixed but changes as the fractional occupancy of receptors changes. With increasing fractional occupancy the affinity for hormone decreases (De Meyts). The negative cooperativity appears to be an intrinsic native characteristic of all insulin receptors studied. Studies are now being pursued to characterize this interaction in detail and to elucidate the molecular mechanisms involved.

Whereas acute changes in insulin concentration regulate the affinity of the receptor for hormone, chronic levels of the circulating hormone, chronic levels of the circulating hormone regulate the number of receptors on the target cell. This regulation of receptor number, which was first demonstrated by us for insulin (Kosmakos) and for growth hormone (Lesniak) and shown by others to apply to catecholamines as well as other peptide hormones, seems to be another general mechanism by which the target cell regulates its sensitivity. Studies thus far indicate that chronic elevations in circulating hormone accelerate degradation of receptor and its disappearance from the plasma membrane. Studies are now in progress to characterize the cellular mechanisms by which loss of target cell receptors occurs as well as those mechanisms by which target cell receptors are restored following removal of the hormone.

We have studied the characteristics of insulin receptors on cells from people with a variety of disorders. For these studies we have largely used the circulating mononuclear cells prepared from buffy coats of freshly drawn blood. We have extended previous work in obese patients where it had been known that obesity and insulin resistance were associated with a decreased number of insulin receptors and chronic dietary restriction restored not only insulin sensitivity but also restored to normal the number of insulin receptors per cell (Archer). Present studies have shown that total fasting for 48-72 hrs. restores insulin binding to normal, at least over the physiological range of insulin concentrations (Bar). This is similar to results that have been previously obtained in obese animals where starvation was associated with a fall in the circulating insulin levels and a restoration of insulin receptor concentrations to normal levels (Kahn). Recent studies have characterized the monocyte as the major insulin binding cell in the mixed mononuclear preparations (Bianco, Kahn). To extend these studies more widely, we have developed specific methods for preparing granulocytes as a receptor system, and have demonstrated normal insulin receptors on normal circulating granulocytes (Fussganger). With white cells we measure the acute effects of the *in vivo* situation on the insulin receptors; in an attempt to measure the fixed characteristics of the cell we have studied insulin receptors on cultured fibroblasts and have applied this to a peculiar disease state in females. These patients all have extreme degrees of insulin resistance, hyperinsulinemia, and a severe deficiency in insulin receptors on their target cells. Cultured fibroblasts from one of these patients have normal complements of insulin receptors suggesting that the defect in insulin binding is not a fixed permanent character of these cells (Rechler).

We have also extended our studies of insulin receptors and fibroblasts to problems of tumor growth. In collaboration with Dr. Ira Pastan and his colleagues, who have developed an extensive set of fibroblast lines with a variety of growth characteristics, we have studied changes in insulin receptors on these cells as a function of cell cycle, cell growth, degree of transformation and of inhibition of growth with increasing density (Thomopoulos).

In addition to using hormones to characterize receptors we have used receptors to characterize hormones. We have fractionated the circulating insulin and circulating growth hormone in serum from patients of many disease states and used the receptors on known target cells to characterize these circulating materials in great detail (Lesniak, Gavin, Gorden). Further, we have used the NSILA-s receptor of the rat liver to measure circulating levels of this insulin-like peptide in a variety of disease states and in response to physiological stimuli. This peptide, which is unmeasurable by conventional radioimmunoassays, can now be measured precisely at physiological concentrations and its special physiology elucidate (Megyesi, Kahn).

Other closely related projects are: studies of the insulin receptor of the avian erythrocyte to study the interactions of insulin with its receptor in a cell that is metabolically simple and intact (Ginsberg); characterization of the insulin receptor of the placenta (Podskalny); isolation and characterization of another insulin-like peptide obtained from tumors of rat livers (Rechler); and an ongoing long-term study on methods for treating acromegaly (Eastman).

Project No. Z01 AM 47,000 - 02 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Cooperative Mechanisms Regulating Receptor's Affinity for Insulin

Previous Serial Number: NIAMDD/CEB 22C

Principal Investigators: Pierre De Meyts, M.D., Jesse Roth, M.D.

Other Investigators: Emmanuel Van Obberghen, M.D., David M. Neville, Jr., M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH

People Years:

Total: 1-1/2

Professional:

Other:

Project Description:

We have extended our studies on the negative cooperativity of insulin receptors. Our previous studies indicated that when insulin binds to its receptors, the overall affinity of the receptors decreases when occupancy increases, due to acceleration in the dissociation of insulin from the receptors (negative cooperativity). We have designed a more precise quantitative analysis of the site-site interactions and described a plausible mechanism. Kinetic data indicate that with increasing occupancy, insulin receptor sites are switched reversibly from a "high affinity state" in which insulin dissociates slowly ("empty sites" conformation) to a "low affinity", fast dissociating state ("filled sites" conformation). The proportion of sites in each state for a given occupancy depends on pH, temperature and ionic strength. In dealing with such site-site interactions, classical methods of steady-state analysis do not yield physically meaningful parameters. In the Scatchard plot of bound/free (B/F) versus bound (B), only the abcissa intercept keeps its physico-chemical meaning of binding capacity or R_o. We propose a new parameter, K_{app}, for describing site-site interactions, where $K_{app} = (B/F)/(R_o - B)$ and is the "apparent average affinity" of the sites at a given fractional saturation. Plotting K_{app} against the fractional saturation (B/R_o) reveals that: (1) at very low occupancy a limiting high K_{app} is obtained ("empty sites" conformation);

(2) K_{app} begins to fall at an occupancy as low as 1 - 5% ("threshold"); (3) above 25% occupancy, a limiting low K_{app} is obtained ("filled sites" conformation). From this analysis, it is apparent that most of the displacement of [^{125}I]-insulin by low concentrations of unlabeled insulin reflects negative cooperativity and not competition. Furthermore, the difference between the two extreme values of K_{app} in this "two state" model is much smaller than the difference between the "high" and "low" affinities of a model with two discrete classes of binding sites with differing affinities. Thus, the same range of sensitivities which would require the cell to synthesize two structurally distinct receptor molecules can be achieved with one molecular species endowed with the ability to undergo induced-fit conformational changes.

As a side project, we have looked at this effect, and at the characteristics of the insulin receptor in general, in a phylogenetic perspective.

Human and other mammalian insulins are very similar in amino acid sequence and equipotent biologically. The exceptions are insulins of guinea-pig, and other histricomorphs, which differ by 33% from the human sequence and are biologically much less potent. In contrast, insulins of birds which differ 14% from the human sequence, are 2 - 3 times more potent than human insulin. In all cases the potencies correlate with their affinities for mammalian receptor. In the present study we examined the insulin receptor of man, rat, mouse, guineq-pig, chicken, and turkey in their reaction with insulins from man, pig, fish, guinea-pig and chicken sources. Irrespective of the species of tissue tested, the insulin receptors were identical in all respects, including specificity and negative cooperativity. All species bound chicken insulin > pork > fish >> guinea-pig. Bird insulin was recognized much better than the others by the receptor of all species, whereas the guinea-pig recognized its own insulin much more poorly than the other insulins. Despite marked evolutionary changes in insulin, the receptor has remained remarkably constant in its physico-chemical properties and recognition of hormone structure. Furthermore, conservation of the negative cooperativity indicates the importance of this mechanism in the action of insulin.

The negative cooperativity of hormone receptors (confirmed since our original description for other receptors like TSH, nerve growth factor and catecholamines) appears thus to be a widespread regulatory mechanism. Our intent is to pursue the study of this mechanism and establish correlations between the binding data and morphological changes in the cell surface.

Keyword Descriptors: Insulin, Receptors, Hormone receptors, Cell membrane receptors, Hormone action, Cooperativity, and Radioreceptor assay.

Honors and Awards: Solomon A. Berson Research and Development Award, American Diabetes Association.

Publications:

1. De Meyts, P. Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. Methods in Receptor Research, M. Blecher, ed., Methods in Molecular Biology, A. I. Laskin and J. A. Last, eds., (1975) in press.

2. De Meyts, P. Cooperative properties of hormone receptors in cell membranes. *J. of Supramolecular Structure*, (1975) in press.

3. Limbird, L. E., De Meyts, P., and Lefkowitz, R. J. β-Adrenergic receptors: evidence for negative cooperativity. *Biochem. Biophys. Res. Commun.*, (1975) in press.

Project No. Z01 AM 47,001-02 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Regulation of Receptors on Cultured Human Lymphocytes by Polypeptide Hormones

Previous Serial Number: NIAMDD/CEB 24C

Principle Investigators: Maxine A. Lesniak, Jesse Roth, M.D., Frank Kosmakos, Ph.D

Other Investigators: Richard Eastman, M.D., David M. Neville, Jr., Phillip Gorden, M.D., Pierre De Meyts, M.D., and David Rabin, M.D.

Cooperating Units: NIMH

People Years:

Total: 4-1/4

Professional: 4

Other: 1/2

Project Description:

I. Chronic exposure of cultured lymphocytes to low, moderate, and high concentrations of hGH at 37° produces a decrease in growth hormone receptor concentration parallel to hormone concentration used. This decrease in GH binding sites does not correlate with competition of same concentration in radioreceptor assay, i.e. 2 x 10^{-10} M hGH causes 50% decrease in binding sites when preincubated with cultured lymphocytes and 10% inhibition of labeled hGH in the radioreceptor assay. This is a reversible phenomenon, when GH is removed from the medium the receptor concentration returns to near normal concentration after 24 hrs. The addition of cycloheximide interferes with reappearance of the GH receptors but not with viability of the cultured cells. Acute exposure of cells to GH has no effect.

II. Chronic exposure of cultured lymphocytes to low, moderate and high concentrations of insulin at 37°C *in vitro* produced a decrease in insulin receptor sites per cell; acute exposure of cells to insulin had no effect. There appears to be a correlation between hormone concentration and time for receptor disappearance to reach a steady state. The loss of receptors is reversible with time by removing the hormone or in part by degradation of the hormone in the medium. Occupancy of the insulin receptor versus loss of insulin receptor has been studied by subjecting the cells to conditions, i.e. change in pH, change in temperature, which would maximize dissociation of the hormone-receptor complex. The results indicate loss of receptor rather than occupancy of the receptor.

Key Word Descriptors: Radioreceptor assay, Cultured human lymphocytes, Regulation of receptors, Insulin, Human growth hormone, Hormone-Receptor complex.

Awards: None

Publications: None

Project No. Z01 AM 47,002-03 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Interaction of insulin with circulating monocytes in normal volunteers and in clinical states of insulin resistance

Previous Serial Number: None

Principal Investigators: Robert Bar, M.D., Phillip Gorden, M.D., C. Ronald Kahn, M.D., and Juanita A. Archer, M.D.

Other Investigators: Jesse Roth, M.D., Maxine Lesniak, A. Rafaele Bianco, M.D., Carla Hendricks, Carolyn Siebert, Jeffrey Flier, M.D., and B. Handwerger, M.D.

Cooperating Units: Istituto di Clinica Medica Generale, Italy, and University of Minnesota, Department of Immunology

People Years:

Total: 6

Professional: 2-1/2

Others:

Project Description:

Insulin interaction with human monocytes has been studied using ^{125}I-insulin and Ficoll-Hypaque preparations of mononuclear peripheral leukocytes as a source of monocytes. Hormone tracer concentrations were in the low physiologic range. Binding of ^{125}I-insulin to monocytes was a rapid and reversible process that was pH and temperature dependent. The optimal pH for binding was 8. At 22°C steady state was reached in 3 hours. When binding studies were performed at 22°, pH 8 with an incubation time of 3 hours, no degradation of hormone or receptor was detected and insulin binding was a linear function of monocyte concentration. Under similar incubation conditions, the ability of insulin analogues to compete for insulin binding sites was directly proportional to the analogue's bioactivity, as measured by glucose oxidation stimulation in adipocytes. Dissociation studies of hormone-receptor complexes demonstrated negatively cooperative interactions at a time when only a minority of binding sites are occupied.

In normal volunteers, insulin binding to mononuclear leukocytes showed little variation when binding was expressed as a function of monocyte concentration. Equilibrium analysis of these binding studies revealed an average of

18,000 binding sites per monocyte. The nonlinearity of Scatchard plots, the Hill coefficient of 0.63 and the data from dissociation experiments fit a model consistent with a single class of insulin receptor sites exhibiting negatively cooperative interactions.

These data indicate (1) the similarity between insulin receptors of circulating human monocytes and those described in fat cells, liver cell plasma membranes and cultured human lymphocytes and (2) optimal conditions for study of insulin binding in normal and disease states.

^{125}I-insulin binding to circulating monocytes has also been measured in 5 obese, hyperinsulinemic patients. Three showed maximal binding of insulin that was not significantly different from normal volunteers. However, 2 patients showed clearly decreased binding of ^{125}I-insulin. The two "low binding" obese patients were fasted for 72 hours at which time maximal binding returned to normal. Chronic diet, resulting in weight losses of 25 and 40 kg. respectively also resulted in normalization of maximal binding. Scatchard analysis of competitive binding isotherms performed after the acute 72 hour fast and after chronic weight loss was associated with an increase of receptor number without significant change in affinity.

Keyword Descriptors: Insulin, Receptors, Monocytes, Obesity, Radioreceptor assay

Honors and Awards: None

Publications:

1. Roth, J., Kahn, C. R., Lesniak, M. A., Megyesi, K., Neville, Jr., D. M., Gavin, III, J. R., Soll, A. H., Freychet, P., Goldfine, I. D., Bar, R. S., Archer, J. A. Receptors for Insulin NSILA-s and Growth Hormone: Applications to Disease States in Man. Recent Progress in Hormone Research, Vol. 31, _in press_.

2. Kahn, C. R., Bar, R. S., Gorden, P., and Roth, J. The syndrome of Acanthosis Nigricans and severe insulin resistance: A primary insulin receptor defect in man. Clinical Research, Vol. 23, 324, 1975 (Abstract).

3. Schwartz, R. H., Bianco, A. R., Handwerger, B. J., and Kahn, C. R. Demonstration that monocytes rather than lymphocytes are the insulin binding cells in peripheral blood mononuclear leukocytes: implications for studies of insulin-resistant states in man. Proc. Nat. Acad. Sci. USA, _72_: 474 (1975).

4. Archer, J. A., Gorden, P., and Roth, J. Defect in insulin binding to receptors in obese man. Amelioration with calorie restriction. J. Clin. Invest. _55_: 166 (1975).

Project No. Z01 AM 47,003-02 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Mulitplication-Stimulating Activity (MSA): Purification, Biosynthesis, Radioreceptor and Biological Assays

Previous Serial Number:

Principal Investigators: Matthew M. Rechler, M.D., and Judy Podskalny

Other Investigators: S. Peter Nissley, M.D., and David M. Neville, Jr., M.D.

Cooperating Units: Metabolism Branch, NCI, Laboratory of Neurochemistry, NIMH

People Years:

Total: 1

Professional: 1/2

Other:

Project Description:

Multiplication-stimulating activity (MSA)is a small acid-soluble polypeptide that has been isolated from media conditioned by rat liver cell cultures. MSA has nonsuppressible insulin-like activity (NSILA) sulfation factor activity, and is a potent stimulator of cell growth. Using extensively purified MSA, we have developed a radioreceptor assay for MSA in rat liver membranes. The receptor assay was highly specific for MSA. NSILA-s, a closely related polypeptide, competed for ^{125}I-MSA binding with the same potency as MSA itself, insulin and proinsulin were weak competitors, and growth hormone and other polypeptides were inactive. Using this MSA radioreceptor assay as well as biological assays for MSA (i.e., incorporation of [^{3}H]-thymidine into DNA in human and chick embryo fibroblasts), we have been unable to detect any effect of growth hormone or estrogens treatment on MSA production by rat liver cells.

Keyword Descriptors: Rat liver cell culture, Multiplication-stimulating activity (MSA), NSILA-s, Radioreceptor assay, Insulin, Chick embryo fibroblasts, Human fibroblasts.

Honors and Awards: None

Publications: None

Project No. Z01 AM 47,004-05 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Insulin Receptors in Diverse Tissues

Previous Serial Number:

Principal Investigators: Matthew M. Rechler, M.D., Judy Podskalny, Pierre Thomopoulos, M.D., Rolf Fussganger, M.D., Barry Ginsberg, M.D., C. Ronald Kahn, M.D.

Other Investigators: Pierre De Meyts, M.D., Phillip Gorden, M.D., Kathy Baird, Janice Chou, Ph.D., Ira Pastan, M.D., Jesse Roth, M.D.

Cooperating Units: Laboratory of Biomedical Sciences, NICHHD, Laboratory of Molecular Biology, NCI

People Years:

Total: 5-1/2

Professional: 1-1/2

Other:

Project Description:

We have demonstrated insulin receptors in fibroblast cultures and granulocyte preparations, adding to the number of cell types available for studying insulin binding in various disease states. Fibroblast cultures were derived from skin biopsies and examined after several generations in culture. They reflect the genetic endowment of the cells, whereas granulocytes obtained by plasmapheresis and examined without cultivation reflect the immediate physiological state of the donor. Insulin receptors of high affinity and specificity have been appreciated in both systems. In the case of the granulocytes, it was necessary to inhibit extensive insulin degrading activity in order to see specific insulin binding. Insulin binding data obtained in both cell systems gave curvilinear Scatchard plots, attributable to cooperative interactions between receptor sites. Fibroblasts from a patient with the syndrome of extreme insulin resistance and acanthosis nigricans bound ^{125}I-insulin to a normal extent and with normal affinity, in contrast to the marked reduction in insulin binding that had been observed in this patient's circulating mononuclear cells.

This suggests that the defect in this insulin-resistant patient is not a genetically determined abnormality of her insulin receptors.

Insulin promotes the growth of many cultured cells. Since transformed cells show abnormal growth regulation, and since it is known that transformed cells differ from normal cells in several surface membrane properties, it was of interest to compare insulin binding to a variety of normal and transformed rodent fibroblast cultures. Transformed cells and growing non-transformed cells appear to have a significant decrease in insulin binding compared to stationary non-transformed cells when compared on the basis of either cell number or cell surface area.

Insulin receptors also have been demonstrated and characterized in a new cell line that recently has been established from normal human placenta. These cells synthesize the α-subunit of chorionic gonadotropin, small amounts of intact gonadotropin, and placental alkaline phosphatase. It is hoped that further studies with this model system will help clarify the role of insulin in placental physiology.

Insulin receptors have been characterized in a non-mammalian tissue, the turkey erythrocyte. Insulin binding to turkey erythrocytes is virtually identical to previously described mammalian receptors with respect to kinetics, specificity, pH dependence, etc. In addition, negative cooperativity among insulin receptors, first demonstrated in mammalian systems, also is present in the turkey erythrocytes. Thus, both the binding properties and the cooperative properties of insulin receptors have been highly conserved in evolution.

Keyword Descriptors: Radioreceptor assay, Human fibroblast culture, Human granulocytes, Insulin resistance, Mononuclear cells, Rodent fibroblasts culture, Human placenta culture, Insulin, Turkey erythrocytes.

Honors and Awards: None

Publications: None

Project No. Z01 AM 47,005-03 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Studies of Nonsuppressible Insulin-like Activity (NSILA-s) of Plasma

Previous Serial Number: NIAMDD/CEB 25C

Principal Investigators: Klara Megyesi, M.D., C. Ronald Kahn, M.D., and Jesse Roth, M.D.

Other Investigators: Phillip Gorden, M.D., David M. Neville, Jr., M.D.
E. R. Froesch, M.D., and R. E. Humbel, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH, Department of Medicine and Institute of Biochemistry, University of Zurich, Switzerland

People Years:

Total: 1-1/2

Professional: 1-1/2

Other:

Project Description:

NSILA-s is a partially purified serum peptide with insulin-like bioactivity but not immunoreactivity. We had previously demonstrated that liver plasma membranes possess separate receptor sites for NSILA-s and insulin and had used the NSILA-s receptor to develop a specific and sensitive radioreceptor assay for plasma NSILA-s. In our present studies we have further characterized the NSILA-s receptor and extended our observations on measurements of plasma NSILA-s in man.

^{125}I-NSILA-s binds rapidly and reversibly to its receptors in liver. The optimum temperature is 20° and the pH optimum is broad. Unlabeled NSILA-s is most potent in displacing the labeled NSILA-s while proinsulin and insulin are less than 1/1000th as potent. Six preparations of NSILA-s which varied 70-fold in biological activity competed for ^{125}I-NSILA-s binding in order of their potency. A purified preparation of MSA (multiplication-stimulating activity), another insulin-like peptide was fully as active as NSILA-s in competing for the NSILA-s receptor. Fibroblast growth factor, epidermal growth factor, nerve growth factor and somatomedin B were less than 1% as effective as NSILA-s in competing for the receptor.

Further studies of NSILA-s in plasma reveal elevated NSILA-s levels in about 5 of 15 patients with extrapancreatic tumors and hypoglycemia. Levels are also elevated in pregnancy and subnormal in hypopituitarism and anorexia nervosa. In hypopituitary patients NSILA-s rises after i.v. hGH. In normal subjects, NSILA-s also rises after oral glucose and falls with insulin induced hypoglycemia. In plasma at physiologic pH about 93% of endogenous NSILA-s is reversibly bound to larger molecules.

Keyword Descriptors: NSILA-s, Insulin, Liver plasma membranes, Radioreceptor assay, Extrapancreatic tumors, Pregnancy, Hypopituitarism, Anorexia nervosa, Hypoglycemia

Honors and Awards: None

Publications:

1. Megyesi, K., Kahn, C. R., Roth, J., and Gorden, P. Circulating NSILA-s in Man: Preliminary studies of stimuli *in vivo* and of binding to plasma components. J. Clin. Endocrin., in press.

Project No. Z01 AM 47,006-03 DB
1. Diabetes Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Radioreceptor Assay of Insulin and Growth Hormone Components

Previous Serial Number: None

Principal Investigators: Maxine Lesniak, Phillip Gorden, M.D., Jesse Roth, M.D., C. Ronald Kahn, M.D.

Other Investigators: Richard Eastman, M.D., James R. Gavin, III, M.D., Carla Hendricks

Cooperating Units: Laboratory of Neurochemistry, NIMH

People Years:

Total: 1-1/2

Professional: 1

Other: 1/2

Project Description:

Previous experiments have demonstrated that the ability of a given insulin or insulin analog to compete for binding sites on cultured human lymphocytes or highly purified rat liver plasma membranes is directly proportional to the biologic activity of the preparation in an independent biologic assay system. This competitive binding system therefore measures a specificity that closely parallels the biologic activity of the preparation and may be quite distinct from the immunologic potency of the preparation. The present experiments demonstrate that proinsulin and its analogs have the same biologic specificity with respect to the lymphocyte and liver membranes as insulin and its analogs. Porcine proinsulin has about 5% of the activity of insulin in stimulating glucose oxidation in fat cells. When the circulating immunoreactive insulin components are separated by filtration on Sephadex G-50 the insulin-like component competes for binding sites on cultured lymphocytes with the same potency as the porcine insulin standard whereas the proinsulin-like component is much less active and has only about 16% of the potency of the porcine insulin standard. The circulating human proinsulin-like component, however, from normal subjects, obese subjects, and subjects with islet cell tumors has a greater potency than porcine proinsulin in the receptor assay. Since purified human proinsulin and porcine proinsulin would be expected to have the same biologic activity, these data further suggest that the circulating proinsulin-like component itself is heterogeneous and probably consists of intermediates or other as yet to be described components which have greater activity than proinsulin.

Analogues to the biologic specificity of the lymphocyte insulin receptor, the cultured lymphocyte growth hormone receptor demonstrates a similar biologic specificity. When the discrete plasma immunoreactive circulating growth hormone components are separated on G-100 Sephadex the "little" growth hormone component has similar receptor and immunologic potency, whereas the "big" growth hormone component has only about 20% of the receptor potency of the "little" component. Further study has demonstrated that the "little" component may be different in different disease states, for instance when 22,000 MW "little" circulating growth hormone component is isolated from acromegalic patients and a similar component is isolated under identical conditions from normal subjects, the receptor activity from the acromegalic subject is 30% greater than from the normal subject. Thus, the acromegalic patient not only has a higher proportion of the more active "little" component, but also has a more receptor-active "little" circulating growth hormone component. Work from several other laboratories has basically confirmed these findings. The radioreceptor assay for growth hormone is particularly useful since biologic assays are at least three to four orders of magnitude less sensitive and are totally impractical for studying plasma growth hormone.

Keyword Descriptors: Radioreceptor assay, Insulin, Growth Hormone, and Rat liver membranes

Honors and Awards: None

Publications:

1. Gorden, P., Lesniak, M. A., Hendricks, C. M., Roth, J., McGuffin, W., and Gavin, J. R., III. Human growth hormone: Characterization of plasma and pituitary components and application of a new radioreceptor assay. Israel J. Med. Sci. 10: 1239-46, 1974.

2. Gorden, P., Hendricks, C. M., Roth, J., and Kahn, C. R. The Plasma proinsulin-like components. Israel J. Med. Sci. 10: 1212-21, 1974.

3. Roth, J.: In Colowick, S. P. and Kaplan, N. O. (Eds) Methods in Enzymology, Academic Press, New York, 1975, Vol. 37, pp. 66.

4. Gorden, P., and Hendricks, C. M. The measurement of plasma insulin and proinsulin-like components. In Croll, M. N., Wallner, R., Honda, T. (Eds.) New Techniques in Tumor Localization and Radioimmunoassay, John Wiley & Sons, New York, 1974, pp. 17-24.

5. Gavin, J. R., III, Kahn, C. R., Gorden, P., Roth, J., and Neville, D. M., Jr Radioreceptor assay of insulin: Comparison of plasma and pancreatic insulins and proinsulins. J. Clin. Endocrinol. Metab. (in press).

PEDIATRIC METABOLISM BRANCH

Cystic Fibrosis of the Pancreas (CF) and Respiratory Distress Syndrome (RDS) have been the objects of investigation by the PMB in 1974.

A. CYSTIC FIBROSIS OF THE PANCREAS (CF)

I. Biochemical and Metabolic Studies

a) Blood polyamine studies

Alteration in blood polyamines in CF patients and obligatory heterozygotes: Because of suggestions in the literature that blood polyamine levels might be altered in cystic fibrosis, spermidine and spermine have been measured in whole blood extracts from a total of 74 individuals, including controls, homozygotes, and obligatory CF heterozygotes. Average ages and ranges (in years) were as follows: 1) controls: male 28, 19-58 (only one individual above 35 years); females 20, 19-35; 2) CF patients: male 17, 2-27 (only 4 individuals below 17 years); female 21, 12-30; 3) CF obligatory heterozygotes: male 35, 26-46; female 39, 24-60. Blood was collected simultaneously for differential blood counts and polyamine analysis. With the exception of one CF patient and one CF heterozygote, who had slightly abnormal leukocyte patterns, blood counts were all in the normal range. Blood was processed within 30 min of collection and the polyamines separated and quantitated by the method of Tabor *et al*. All data were analyzed according to age, sex, and genotype (relative to CF). No age relationship was apparent; however, a significant sex difference was found, with control females showing a mean blood Spm ($p<0.05$ as compared to control males) and a higher Spd/Spm ratio ($p<0.05$). For this reason, data are reported and analyzed according to sex.

As seen in the table below, male homo- and heterozygotes for CF exhibit a consistent and significant decrease in blood spermine resulting in an elevated spermidine/spermine ratio when compared to control males.

BLOOD POLYAMINE CONCENTRATIONS AND RATIOS IN MALES

Mean figures, expressed as nmoles/ml whole blood ± S.E.M., are accompanied by the standard error of the mean and the p value as compared to the control group and as determined by the paired t test.

	Male controls (n = 14)	Male CF (n = 14)	Male CF Hz (n = 7)
Spermidine	10.30 ± 1.01	9.24 ± 0.56 (<0.4)	10.31 ± 1.12 (<0.9)
Spermine	9.97 ± 0.87	6.57 ± 0.54 (<0.005)	6.79 ± 0.67 (<0.05)
Ratio (Spd/Spm)	1.05 ± 0.06	1.48 ± 0.09 (<0.001)	1.53 ± 0.16 (<0.005)

In contrast, control females exhibited reduced spermidine and spermine levels as compared to control males, and female CF homo- and heterozygotes showed similar results. It was felt that the sex difference was probably due to fluctuations of blood polyamine levels during the menstrual cycle.

A definite answer regarding the question of whether or not a disturbance of polyamine metabolism could play a pathogenetic role in CF cannot be given at this time. However, it is of interest to note that glycoprotein metabolism, which is believed by many to be disturbed in this disorder, can be influenced by spermine and that at least in bacteria, the polyamines may play an important role in maintaining an appropriate salt balance across the cellular membrane. It will be recalled that the most consistent chemical finding in CF individuals is the high concentration of sweat sodium, chloride, and potassium. Anyway, primary or secondary as these findings may be in this disorder, they represent the first consistent abnormality of a low molecular substance which can be defined chemically and which is present in both homo- and heterozygotes for the CF gene. (Dr. D. Lundgren, Dr. P. Farrell, Dr. P. di Sant'Agnese, NIAMDD).

Influence of the menstrual cycle on blood polyamine levels: Because of the difference observed between male and female CF patients and heterozygotes in blood polyamine levels which is in conflict with the accepted autosomal recessive mode of inheritance of CF and the possibility that sex-related hormones might be influencing the whole blood Spd/Spm ratio, these investigations were extended.

Whole blood Spd/Spm ratios were determined in 3 normal males, 3 normal females, and 1 ovariectomized female over a 4-week period.

BLOOD SPERMIDINE/SPERMINE RATIOS FOR MALE AND FEMALE CONTROLS
STUDIED OVER A 4-WEEK INTERVAL

		Spermidine/Spermine		
		Highest Ratio	Mean Ratio ± SEM	Lowest Ratio
Males	1	1.19	1.06 ± .07*	.83
	2	1.03	1.01 ± .01*	.97
	3	1.31	1.11 ± .08*	.82
Females	1	1.63	1.22 ± .07	.90
	2	1.78	1.37 ± .10	1.06
	3	1.79	1.21 ± .16	1.03
Ovariectomized Female		1.30	1.17 ± .04**	1.01

*p<.02 as compared to the male cystic fibrosis Spd/Spm ratio.

**p<.05 as compared to the male cystic fibrosis Spd/Spm ratio.

As can be seen from the table, individual male Spd/Spm ratios and polyamine concentrations exhibited very little variance from one sampling to the next. In contrast, the normal female ratios and concentrations fluctuated substantially over this time frame. The ratio appeared to increase as the first rise in plasma estradiol occurred during the menstrual cycle and fell as plasma progesterone increased. The ovariectomized female's Spd/Spm ratio pattern was characteristic of males. In addition, one of the four normal females received an oral contraceptive after the initial studies and was followed for an additional four weeks, after which her Spd/Spm ratio profile was characteristic of males. Additional studies are being performed in animals to further determine the influence of progesterone and estradiol on blood polyamine levels.

It appears as though a sex-related factor(s) does influence the female Spd/Spm ratio during the menstrual cycle. These results provide an explanation why male, but not female CF patients displayed an elevated ratio in our previous studies. It has been concluded that female Spd/Spm ratios should be examined at a specific time within the menstrual cycle to define the Spd/Spm ratio of the normal female population before a comparison can be made to female CF patients. (Dr. D. Lundgren, Dr. P. Farrell, Dr. L. Cohen, J. Hankins, NIAMDD).

Localization of spermidine and spermine in formed elements of blood of cystic fibrosis patients and control subjects: This study was prompted by a recent finding in our laboratory that whole blood samples from 47 CF homo- and heterozygotes (Hz) show lower Spm and a higher Spd/Spm ratio than 27 matched controls. Although blood polyamines (PA) represent the first chemically definable components consistently abnormal in CF-Hz, their localization as to the specific cell type remained to be defined in normal and pathological metabolism.

Accordingly, seven (3 control, 4 CF) blood fractionations have been performed by a modification of Rabinowitz's glass bead technique and Ficoll-Hypaque gradients resulting in essentially pure fractions of plasma, RBCs, lymphocytes (Ly), and granulocytes (Gr). PA were extracted with perchloric acid, separated and quantitated via an amino acid analyzer. Results indicate that plasma contains <0.5 nmole, PA/ml (<5% of whole blood PA) and that the cellular distribution of Spd and Spm, respectively, is as follows (nmole/10^9 cells): RBC 1.65±.34, 0.74±.13; Ly 149±21; 452±101; Gr <5, 254±94. Despite the higher cellular content in WBCs, conversion of Spd and Spm levels to a unit blood volume basis reveals that red cells account for >90% of circulating PA. CF blood was again found to have a higher ($p<.01$) Spd/Spm ratio than control samples (2.06±.34 vs. .82±.1). Of great interest and in keeping with the distribution data, purified RBCs from CF patients also show an elevated ratio (CF = 3.02±.44; control = 1.53±.39).

It is concluded that the major portion of blood PA spermidine and spermine are present in the RBC fraction and that blood Spd/Spm abnormalities in patients with CF are reflected in CF red cells. (Dr. L. Cohen, Dr. P. Farrell, J. Willison, Dr. D. Lundgren, NIAMDD).

b) Effects of cystic fibrosis serum on ciliary activity

In serum of both homo- and heterozygotes for CF a factor has been found which causes ciliary dyskinesia in rabbit tracheal cilia *in vitro* or cessation of beat in the cilia of oyster gills. Both of these bioassays are subjective, difficult to interpret and reproduce for a variety of reasons, and have severe limitations not only from a practical standpoint of determining heterozygosity, but also from an investigational standpoint.

In an effort to develop a quantitative chemical assay we have tried a fresh approach by measuring motility-coupled ciliary ATP hydrolysis in the presence of serum. In addition, to characterize the ATPase activity several basic studies were performed with oyster gill and rabbit tracheal cilia. Homogeneous, motile cilia were prepared from the former by mincing in 0.05 M Tris HCl (pH 8.0), 0.3 mM EDTA; this preparation, which on electron microscopy showed intact cilia, hydrolyzed ATP in proportion to calculated beat frequency. A high molecular weight ATPase was purified from rabbit tracheal cilia and shown to have properties similar to flagellar dynein. The effect of various factors (time, temperature, pH, cations, and substrate concentration) on ciliary ATP hydrolysis was determined. Explants, homogenates, and minces of rabbit trachea and oyster gill were preincubated for 30-45 min with either normal, CF, or CF heterozygote sera; subsequent addition of ATP-γ-^{32}p and measurement of ^{32}Pi production revealed that the latter two groups could not be distinguished from normal serum. Studies are continuing but this objective, quantitative assay has thus far failed to demonstrate the "CF factor." (Dr. P. Farrell, G. Fox (guest worker), NIAMDD; Dr. S. Spicer, Medical Col. of S. Carolina, Charleston).

c) Vitamin E in man

Vitamin E deficiency in man: Man has not been shown to develop clinical symptoms from vitamin E deficiency, although protean manifestations of tocopherol deficiency occur in animals. Patients with cystic fibrosis provide the best available model of vitamin E deficiency in a controlled human population, especially as previous investigations have been hampered by the small populations of subjects available for investigation.

Accordingly, studies were carried out on the degree and effects of tocopherol deficiency in a group of 50 patients with pancreatogenous steatorrhea due to cystic fibrosis. The results of plasma α-tocopherol determinations revealed that the subjects are uniformly deficient in vitamin E. The degree of diminution varied considerably but correlated in general with biochemical indicators of intestinal malabsorption. In particular, it was found that plasma α-tocopherol levels were proportional to serum carotene and cholesterol concentrations; changes in these three substances were inversely proportional to fecal fat excretion. In contrast to the subjects with steatorrhea, one cystic fibrosis patient with normal pancreatic function was found to have a normal plasma α-tocopherol concentration. In addition, a number of subjects receiving dietary supplements of water miscible vitamin E (1-10 IU/kg/day) were evaluated in terms of plasma tocopherol concentration. This group exhibited plasma levels of the vitamin which were at least as high as normal and in some cases elevated in comparison to control subjects. Tocopherol concentration was also measured in erythrocytes from CF patients in a limited number of tissues obtained at autopsy. These samples showed diminished levels in agreement with plasma analyses.

Studies on the effects of vitamin E deficiency focused on possible hematologic alterations but also included an assessment of skeletal muscle as well as examination of subcellular membranous organelles via electron microscopy. Erythrocyte hemolysis was measured both *in vitro*, with an improved peroxide susceptibility test, and *in vivo* using survival of ^{51}Cr-labeled red cells as the primary index. With the use of 2% hydrogen peroxide under gentle conditions of washing and mixing, it was found that <1% of erythrocytes in suspensions from control subjects underwent hemolysis during a 3-hr incubation at 37°C. In contrast, unsupplemented cystic fibrosis patients with pancreatic achylia showed 80% hemolysis on the average, a range of 17-96%. The stability of erythrocytes to peroxide challenge related to plasma α-tocopherol in a sigmoidal manner. Plasma α-tocopherol levels of <100 μg/100 ml were associated with >90% hemolysis, whereas samples with >500 μg/100 ml were almost completely resistant to oxidant damage. The survival of autologous ^{51}Cr-labeled red cells was determined in 19 vitamin E deficient patients and found to be significantly ($p<.005$) decreased to 22.4 days in comparison to control subjects who showed a mean ^{51}Cr-$t_{1/2}$ of 27.0 days. Erythrocyte survival was measured in 6 subjects before and after dietary tocopherol supplementation. While in the vitamin E deficient state this group showed a shortened RBC life span with a mean ^{51}Cr-$t_{1/2}$ of 19.1 days, whereas after supplementation the figure rose significantly ($p<.001$) to 27.6 days. Static hematologic indices, however, such as red cell count, hemaglobin concentration, and hematocrit value, were generally normal and did not rise consistently in patients supplemented with vitamin E. Thus, as the ^{51}Cr-RBC data suggest, the degree of hemolysis *in vivo* is minimal and not sufficient to cause frank hemolytic anemia.

Measure of urinary creatine excretion was carried out in an attempt to assess muscle function. Excessive creatinuria was observed in nearly all the 25 subjects so evaluated; however, this index did not consistently improve upon supplementation with vitamin E. Furthermore, only two of 40 vitamin E deficient subjects showed elevations in muscle-derived plasma enzymes, such as creatine kinase and aldolase. In addition, skeletal muscle samples obtained by biopsy from 5 subjects failed to reveal evidence of gross myopathy. Thus, the creatinuria is likely attributable to chronic wasting rather than acute or sub-acute muscular degeneration. Morphological studies on biopsy-procured jejunal mucosa revealed no evidence of ultrastructural changes. In particular, subcellular membranous organelles were preserved in vitamin E deficient patients.

It is concluded that vitamin E deficiency is regularly associated with cystic fibrosis providing such patients have pancreatic involvement and are not taking supplementary doses of the vitamin. Concomitant hematologic effects consistent with mild hemolysis, but not anemia, are found in these patients but may be reversed upon vitamin E supplementation.

Megavitamin E supplementation in man: To assess possible toxicity or beneficial effects of vitamin E, a group of 28 adults voluntarily ingesting 100 to 800 IU/day of vitamin E for an average of 3 yrs were evaluated in this study.

Half of the subjects claimed a feeling of improved health or wellbeing, but no specific beneficial effects were noted consistently. Half of the subjects indicated no change in health status after beginning vitamin E supplements. No gross evidence of toxicity was apparent upon reviewing the past medical history with the subjects. Plasma α-tocopherol was found to be elevated significantly in the group, from 740 μg/100 ml (control mean) to 1340 μg/100 ml. However, 25% of the values were within 2 S.D. of the control mean, despite repeated claims that these subjects were taking the originally indicated dose. Plasma α-tocopherol levels did not correlate with total daily dose but did relate to plasma triglyceride and cholesterol concentrations. Total plasma carotenoids were increased significantly along with vitamin A levels; the former did not correlate with plasma vitamin E, whereas the latter showed a significant correlation. Laboratory screening for toxic side effects of the vitamin by performance of 22 standard clinical blood tests failed to reveal any disturbance in liver, kidney, muscle, thyroid gland, erythrocyte, leukocytes, coagulation parameters, or blood glucose. It is concluded that megavitamin E supplements in this group produced no apparent toxic side effects and that subjective claims for beneficial effects were highly variable.

Conclusions: The metabolic role of this ubiquitous biological antioxidant is still unclear, but its widespread presence in human tissues must serve a purpose. Patients with malabsorption and secondary tocopherol deficiency should, therefore, receive supplements of vitamin E. Such supplementation should also be considered in the dietary makeup of populations in areas of the world (e.g., Bangladesh, Jordan, etc.) suffering from malnutrition and receiving inadequate diets. However, there is little reason to add vitamin E supplements in countries such as the United States where people ingest adequate amounts of the vitamin in their diets and have normal blood tocopherol levels. (Dr. P. Farrell, Dr. J. Bieri, Dr. P. di Sant'Agnese, NIAMDD; Dr. V. Fischer, Univ. of St. Louis, MO).

II. Clinical Studies

a) Pseudomonas vaccine in cystic fibrosis: Colonization of the respiratory tract with Pseudomonas aeruginosa occurs in most patients with cystic fibrosis (80% of the patient population followed at the NIH). This contributes to the morbidity and mortality in CF by increasing bronchial obstruction and leading to bronchiectasis and necrotizing pneumonia. Pseudomonas organisms are notoriously resistant to most of the commonly used antibiotics, and therefore treatment may be difficult. Enhancement of the humoral and cellular pulmonary defense mechanisms of the patients may be another way of approaching the clinical problem of Pseudomonas infection in CF. We have employed a multivalent lipopolysaccharide antigen (Pseudogen, Parke-Davis Co.) to study the antibody responses of 12 CF patients. Each patient was given 6 doses of the vaccine intramuscularly over a two-week period. Although side effects did occur (fever, malaise, local tenderness, and induration) no patient had to be withdrawn from the study because of adverse reactions to the vaccine. Serum hemagglutinin titers to each of the seven serotypes contained in the vaccine were determined before and at intervals following the immunization. Good antibody response, i.e., significant antibody titer increases, were found in all patients. Elevated titers persisted for 3-6 months and then returned to baseline values.

In the small group of patients studied, no objective improvement in pulmonary function or clinical status was noted. Pseudomonas was not eradicated from any patient's sputum. Two patients had what they felt were significant, albeit subjective,improvements for several months following immunization. We conclude from our studies to date that although good antibody responses can be achieved without serious side effects, there is no significant clinical response in patients in whom Pseudomonas colonization is already well established. Clinical trials may be warranted in a larger number of patients, concentrating on those in whom Pseudomonas colonization has not yet occurred. (The intranasal route for immunization would seem well suited for this approach, if it is effective in producing significant antibody responses.) (Dr. R. Wood, NIAMDD; Dr. H. Reynolds, Dr. J. Pennington, NIAID).

b) Mucociliary velocity and transport in cystic fibrosis: Chronic pulmonary disease, secondary to bronchiolar and bronchial obstruction and bacterial infection, is the major cause of morbidity and mortality in cystic fibrosis. It has long been assumed that this was due to increased viscosity of pulmonary secretions with subsequent decreased mucociliary airway clearance. Recently, *in vivo* studies of mucociliary airway clearance have suggested that clearance rates in CF patients were in fact faster than normal (New Eng. J. Med. 288:651, 1973).

We have studied tracheal mucus velocity in patients with cystic fibrosis by a new technique (J. Appl. Physiol. 34:495, 1973). A fiberoptic bronchoscope is passed into the trachea of conscious volunteers under topical lidocaine anasthesia. Teflon discs are then blown onto the tracheal mucosa and their movement filmed with a movie camera. The discs are 0.67 mm in diameter and weigh 0.13 mg, well within the transport capabilities of the mucociliary system. The rate of movement is calculated from the change in image size of each disc with time, and is accurate within 10%. Xylocaine has been shown not to alter mucus velocity in dogs and sheep.

The average rate of mucus velocity in 14 patients with CF (ages 18-43, mean = 25) was 2.6 ± 3.3 mm/min (std. dev.). This was markedly slower than the rate in 20 normal volunteers (ages 18-44, mean = 27): 20.1 mm/min ± 6.4 (std. dev.) ($p<.001$). There was no co-relation of mucus velocity with age, sex, clinical status or pulmonary functions.

Following the measurement of mucus velocity, each subject was given 0.25 mg Terbutaline, a new beta-adrenergic agent; by subcutaneous injection. Fifteen min later the mucus velocity was measured again. There was no significant effect in normal volunteers, but in the CF patients the mucus velocity increased to 5.5 mm/min ± 3.6 mm/min ($p<.001$).

This study is the first to measure by a direct method the rate of mucociliary airway clearance in patients with CF, and to demonstrate its impairment in this disease. Furthermore, it is the first to demonstrate pharmacological improvement of mucus clearance in CF. Both these observations have potential therapeutic as well as theoretical implications, and may lead to a greater understanding and better treatment of cystic fibrosis. (Dr. R. Wood, Dr. P. Farrell, NIAMDD; Dr. A. Wanner, Mt. Sinai Hos. Med. Cen., Miami Beach, Fla.).

B. RESPIRATORY DISTRESS SYNDROME (RDS)

Considerable evidence has accumulated suggesting that atelectasis in RDS develops as a result of deficient pulmonary surfactant, particularly lecithin (Pediatrics 40:13, 1967). In our studies, biochemical regulation of lecithin synthesis has been evaluated in fetal lungs from various species. Recently it has been found that by obtaining samples of amniotic fluid of mothers during gestation and assessing the content of lecithin which rises rapidly in late pregnancy, it is possible to predict the patients who are going to be at risk of RDS at birth. This, of course, is especially so if a mother has previously had a child with RDS so that the subsequent ones are apt to have the same manifestations.

The compound is produced *de novo* by two pathways: the choline incorporation route (I) and phosphatidyl ethanolamine methylation (II).

Our studies have demonstrated that pathway I is predominant and accounts for increased lecithin synthesis in the mature fetus as well as for the surge in amniotic fluid lecithin/sphingomyelin ratio.

Enzymatic synthesis of phosphatidylcholine (PC) in homogeneous cultures of apparent type II pneumonocytes: Accelerated production of PC-rich pulmonary surfactant occurs following treatment with various hormones. To delineate pathways and regulation of PC biosynthesis, investigations are being conducted with diploid epithelial cells isolated from rat lung (clone L-2) which have been shown to contain osmiophilic lamellar (*In Vitro* 10, 1975). Studies to date have employed over 50 cultures of L-2 cells grown in either a basal medium (control) or in the presence of hormones such as thyroxin and cortisol. The three catalysts of the principal *de novo* route for PC synthesis - the choline pathway - were readily detected in control cells and compared in terms of specific enzymatic activities with rat lung homogenates and fibroblasts from lung and skin. Results indicate that L-2 cells are enriched to as high as tenfold in choline kinase (CK), cholinephosphate cytidyl transferase, and cholinephosphotransferase (CPT). Cells continuously cultured in the presence of thyroxin showed markedly increased CK (mean±SE = 4815±353 cpm product/min/mg protein; control = 2858±232) but reduced CPT (both $p<.001$). Although cortisol alone produced only slight enzyme elevations, its addition to thyroxin-supplemented cells led to a seven-fold increase in CPT activity ($p<.001$).

It may be concluded that L-2 cells in tissue culture are 1) capable of active PC production, and 2) respond to hormones in a manner consistent with observations on lungs of treated animals. (Dr. P. Farrell, Dr. D. Lundgren, NIAMDD; Dr. W. Douglas, W. Alton Jones Cell Science Center, Lake Placid, NY).

Correlation of fetal lecithin synthesis and the amniotic fluid (AF) L/S ratio in primates: Rhesus monkey gestations were utilized to examine the relationship between the AF L/S ratio and fetal lung lecithin synthesis. In 24 fetuses (114-161 days, term 164) the rate of lung lecithin synthesis *in vitro* via choline incorporation (I) and phosphatidylethanolamine (PE) methylation (II) was determined by incubating lung slices with 2 μCi of ^{14}C-choline or ^{14}C-methionine. Choline incorporation accounted for 90% of lung lecithin synthesis in all fetuses. Pathway I activity was stable from 114-148 days and then abruptly increased with peak activity (3 x baseline, $p<.001$) at day 150 (90% term). The AF L/S ratio, determined in these and 60 additional gestations (70-161 days) showed a gestational pattern similar to that in humans with an abrupt increase at 90% of term. The correlation between lung choline incorporation and AF L/S ratio in the last 30% of gestation was significant ($r = .77$, $p<.001$); lung PE methylation showed no correlation with the L/S ratio. To confirm that lung choline incorporation into lecithin determines the AF L/S ratio, 1 mCi of ^{14}C-choline was injected into the maternal aorta (2 monkeys, 142 days gestation) to provide labelled precursor for fetal lung ^{14}C-lecithin synthesis. AF ^{14}C-lecithin specific activity was determined every 48 hrs over 2 weeks; it increased gradually until day 150 when an abrupt 2.5 fold increase (5.6 → 12 & 2.8 → 7.4 cpm/μg lecithin) occurred. This was coincident with a 30% and 120% increase in the L/S ratio (3.4 → 4.4 & 2.3 → 5.1). It is concluded that the AF L/S ratio accurately reflects developmental changes in fetal lung lecithin synthesis. (Dr. P. Farrell, J. Willison (Guest Worker), NIAMDD; Dr. M. Epstein, NICHD).

Primate fetal lung in gestations complicated by maternal glucose intolerance: The effects of maternal glucose intolerance on primate fetal lung lecithin synthesis were studied in the Macaca mulatta. Ten monkeys in the first trimester were treated with streptozotocin (Stz, 47 mg/kg IV), a pancreatic beta-cell cytotoxin, with resultant impairment of maternal insulin secretion and glucose clearance in response to IV GTT's. Ten pregnancies, delivered by C-section at 86-89% term, showed changes similar to those seen in human diabetic pregnancies - fetal macrosomia and beta-cell hyperplasia, hydramnios, and placental hyperplasia. *De novo* pulmonary lecithin synthesis via the two major pathways - choline incorporation (I) and phosphatidylethanolamine methylation (II) was measured in fetal lung slices incubated with 2 μCi of ^{14}C-choline or ^{14}C-methionine and the amniotic fluid (AF) L/S ratio was determined. Five fetuses of normal, age-matched gestations were controls.

Results:	Pathway I	Pathway II	AF L/S Ratio
Stz	800 ± 90*	90 ± 35	2.5 ± 0.4*
Normal	490 ± 60	20 ± 1	1.0 ± 0.2

Pathway I activity (cpm/mg lung/hr) accounted for 90-96% of lecithin synthesis in both groups. Pathway I activity and AF L/S ratio were significantly (*$p<.05$) greater in the Stz group; pathway II was not. Therefore, maternal glucose intolerance was associated with an increase in fetal lung choline incorporation into lecithin, reflected in an earlier increase in the AF L/S ratio. This suggests that in the human it may be the timing and mode of delivery rather than maternal diabetes mellitus that results in the increased risk of RDS in the neonate. (Dr. P. Farrell, NIAMDD; Dr. M. Epstein, NICHD).

Project No. Z01 AM 49000-02 PMB
1. Pediatric Metabolism
2.
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Biochemical and Metabolic Studies in Cystic Fibrosis.

Previous Serial No.: NIAMDD-PMB-1c

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., Lawrence F. Cohen, M.D., David W. Lundgren, Ph.D., John G. Bieri, Ph.D., NIAMDD.

Cooperating Units: Laboratory of Nutrition and Endocrinology, NIAMDD; Vernon Fischer, Ph.D., St. Louis Univ. Medical School; Samuel Spicer, M.D., Medical College of S. Carolina, Charleston.

Man Years: Total: 4.0
Professional: 2.0
Other: 2.0

Project Description:

Objectives: To elucidate the basic defect in cystic fibrosis and clarify some of the metabolic effects of inherited inborn error of metabolism.

1) Blood polyamine studies.

Alteration in blood polyamines in CF patients and obligatory heterozygotes: Because of suggestions in the literature that blood polyamine levels might be altered in cystic fibrosis, spermidine and spermine have been measured in whole blood extracts from a total of 74 individuals, including controls, homozygotes, and obligatory CF heterozygotes. Average ages and ranges (in years) were as follows: 1) controls: male 28, 19-58 (only one individual above 35 years); females 20, 19-35; 2) CF patients: male 17, 2-27 (only 4 individuals below 17 years); female 21, 12-30; 3) CF obligatory heterozygotes: male 35, 26-46; female 39, 24-60. Blood was collected simultaneously for differential blood counts and polyamine analysis. With the exception of one CF patient and one CF heterozygote, who had slightly abnormal leukocyte patterns, blood counts were all in the normal range. Blood was processed within 30 min of collection and the polyamines separated and quantitated by the method of Tabor et al. All data were analyzed according to age, sex, and genotype (relative to CF). No age relationship was apparent; however, a significant sex difference was found, with control females showing a mean blood Spm ($p<0.05$) as compared to control males and a higher Spd/Spm ratio ($p<0.05$). For this reason, data are reported and analyzed according to sex.

As seen in the table below, male homo- and heterozygotes for CF exhibit a consistent and significant decrease in blood spermine resulting in an elevated spermidine/spermine ratio when compared to control males.

BLOOD POLYAMINE CONCENTRATIONS AND RATIOS IN MALES

Mean figures, expressed as nmoles/ml whole blood ± S.E.M., are accompanied by the standard error of the mean and the p value as compared to the control group and as determined by the paired t test.

	Male controls (n = 14)	Male CF (n = 14)	Male CF Hz (n = 7)
Spermidine	10.30 ± 1.01	9.24 ± 0.56 (<0.4)	10.31 ± 1.12 (<0.9)
Spermine	9.97 ± 0.87	6.57 ± 0.54 (<0.005)	6.79 ± 0.67 (<0.05)
Ratio (Spd/Spm)	1.05 ± 0.06	1.48 ± 0.09 (<0.001)	1.53 ± 0.16 (<0.005)

In contrast, control females exhibited reduced spermidine and spermine levels as compared to control males, and female CF homo- and heterozygotes showed similar results. It was felt that the sex difference was probably due to fluctuations of blood polyamine levels during the menstrual cycle.

A definite answer regarding the question of whether or not a disturbance of polyamine metabolism could play a pathogenetic role in CF cannot be given at this time. However, it is of interest to note that glycoprotein metabolism, which is believed by many to be disturbed in this disorder, can be influenced by spermine and that at least in bacteria, the polyamines may play an important role in maintaining an appropriate salt balance across the cellular membrane. It will be recalled that the most consistent chemical finding in CF individuals is the high concentration of sweat sodium, chloride, and potassium. Anyway, primary or secondary as these findings may be in this disorder, they represent the first consistent abnormality of a low molecular substance which can be defined chemically and which is present in both homo- and heterozygotes for the CF gene.

Influence of the menstrual cycle on blood polyamine levels: Because of the difference observed between male and female CF patients and heterozygotes in blood polyamine levels which is in conflict with the accepted autosomal recessive mode of inheritance of CF and the possibility that sex-related hormones might be influencing the whole blood Spd/Spm ratio, these investigations were extended.

Whole blood Spd/Spm ratios were determined in 3 normal males, 3 normal females, and 1 ovariectomized female over a 4-week period.

BLOOD SPERMIDINE/SPERMINE RATIOS FOR MALE AND FEMALE CONTROLS STUDIED OVER A 4-WEEK INTERVAL

		Spermidine/Spermine		
		Highest Ratio	Mean Ratio ± SEM	Lowest Ratio
Males	1	1.19	1.06 ± .07*	.83
	2	1.03	1.01 ± .01*	.97
	3	1.31	1.11 ± .08*	.82
Females	1	1.63	1.22 ± .07	.90
	2	1.78	1.37 ± .10	1.06
	3	1.79	1.21 ± .16	1.03
Ovariectomized Female		1.30	1.17 ± .04**	1.01

*$p<.02$ as compared to the male cystic fibrosis Spd/Spm ratio.
**$p<.05$ as compared to the male cystic fibrosis Spd/Spm ratio.

As can be seen from the table, individual male Spd/Spm ratios and polyamine concentrations exhibited very little variance from one sampling to the next. In contrast, the normal female ratios and concentrations fluctuated substantially over this time frame. The ratio appeared to increase as the first rise in plasma estradiol occurred during the menstrual cycle and fell as plasma progesterone increased. The ovariectomized female's Spd/Spm ratio pattern was characteristic of males. In addition, one of the four normal females received an oral contraceptive after the initial studies and was followed for an additional four weeks, after which her Spd/Spm ratio profile was characteristic of males. Additional studies are being performed in animals to further determine the influence of progesterone and estradiol on blood polyamine levels.

It appears as though a sex-related factor(s) does influence the female Spd/Spm ratio during the menstrual cycle. These results provide an explanation why male, but not female CF patients displayed an elevated ratio in our previous studies. It has been concluded that female Spd/Spm ratios should be examined at a specific time within the menstrual cycle to define the Spd/Spm ratio of the normal female population before a comparison can be made to female CF patients.

Localization of spermidine and spermine in formed elements of blood of CF patients and control subjects: This study was prompted by a recent finding in our laboratory that whole blood samples from 47 CF homo- and heterozygotes (Hz) show lower Spm and a higher Spd/Spm ratio than 27 matched controls. Although blood polyamines (PA) represent the first chemically definable components consistently abnormal in CF-Hz, their localization as to the specific cell type remained to be defined in normal and pathological metabolism.

Accordingly, seven (3 control, 4 CF) blood fractionations have been performed by a modification of Rabinowitz's glass bead technique and Ficoll-Hypaque gradients resulting in essentially pure fractions of plasma, RBCs, lymphocytes (Ly), and granulocytes (Gr). PA were extracted with perchloric acid, separated and quantitated via an amino acid analyzer. Results indicate that plasma contains <0.5 nmole, PA/ml (<5% of whole blood PA) and that the cellular distribution of Spd and Spm, respectively, is as follows (nmole 10^9 cells): RBC 1.65±.34, 0.74±.13; Ly 149±21; 452±101; Gr <5, 254±94. Despite the higher cellular content in WBCs, conversion of Spd and Spm levels to a unit blood volume basis reveals that red cells account for >90% of circulating PA. CF blood was again found to have a higher ($p<.01$) Spd/Spm ratio than control samples (2.06±.34 vs. .82±.1). Of great interest and in keeping with the distribution data, purified RBCs from CF patients also show an elevated ratio (CF = 3.02±.44; control = 1.53±.39).

It is concluded that the major portion of blood PA spermidine and spermine are present in the RBC fraction and that blood Spd/Spm abnormalities in patients with CF are reflected in CF red cells.

2) Effects of cystic fibrosis serum on ciliary activity.

In serum of both homo- and heterozygotes for CF a factor has been found which causes ciliary dyskinesia in rabbit tracheal cilia *in vitro* or cessation of beat in the cilia of oyster gills. Both of these bioassays are subjective, difficult to interpret and reproduce for a variety of reasons, and have severe limitations not only from a practical standpoint of determining heterozygosity, but also from an investigational standpoint.

In an effort to develop a quantitative chemical assay we have tried a fresh approach by measuring motility-coupled ciliary ATP hydrolysis in the presence of serum. In addition, to characterize the ATPase activity several basic studies were performed with oyster gill and rabbit tracheal cilia. Homogeneous, motile cilia were prepared from the former by mincing in 0.05 M Tris HCl (pH 8.0), 0.3 mM EDTA; this preparation, which on electron microscopy showed intact cilia, hydrolyzed ATP in proportion to calculated beat frequency. A high molecular weight ATPase was purified from rabbit tracheal cilia and shown to have properties similar to flagellar dynein. The effect of various factors (time, temperature, pH, cations, and substrate concentration) on ciliary ATP hydrolysis was determined. Explants, homogenates, and minces of rabbit trachea and oyster gill were preincubated for 30-45 min with either normal, CF, or CF heterozygote sera; subsequent addition of ATP-γ-^{32}p and measurement of ^{32}Pi production revealed that the latter two groups could not be distinguished from normal serum. Studies are continuing but this objective, quantitative assay has thus far failed to demonstrate the "CF factor."

3) Vitamin E in man.

Vitamin E deficiency in man: Man has not been shown to develop clinical symptoms from vitamin E deficiency, although protean manifestations of tocopherol deficiency occur in animals. Patients with cystic fibrosis provide the best available model of vitamin E deficiency in a controlled human population, especially as previous investigations have been hampered by the small populations of subjects available for investigation.

Accordingly, studies were carried out on the degree and effects of tocopherol deficiency in a group of 50 patients with pancreatogenous steatorrhea due to cystic fibrosis. The results of plasma α-tocopherol determinations revealed that the subjects are uniformly deficient in vitamin E. The degree of diminution varied considerably but correlated in general with biochemical indicators of intestinal malabsorption. In particular, it was found that plasma α-tocopherol levels were proportional to serum carotene and cholesterol concentrations; changes in these three substances were inversely proportional to fecal fat excretion. In contrast to the subjects with steatorrhea, one cystic fibrosis patient with normal pancreatic function was found to have a normal plasma α-tocopherol concentration. In addition, a number of subjects receiving dietary supplements of water miscible vitamin E (1-10 IU/kg/day) were evaluated in terms of plasma tocopherol concentration. This group exhibited plasma levels of the vitamin which were at least as high as normal and in some cases elevated in comparison to control subjects. Tocopherol concentration was also measured in erythrocytes from CF patients in a limited number of tissues obtained at autopsy. These samples showed diminished levels in agreement with plasma analyses.

Studies on the effects of vitamin E deficiency focused on possible hematologic alterations but also included an assessment of skeletal muscle as well as examination of subcellular membranous organelles via electron microscopy. Erythrocyte hemolysis was measured both *in vitro*, with an improved peroxide susceptibility test, and *in vivo* using survival of ^{51}Cr-labeled red cells as the primary index. With the use of 2% hydrogen peroxide under gentle conditions of washing and mixing, it was found that <1% of erythrocytes in suspensions from control subjects underwent hemolysis during a 3-hr incubation at 37°C. In contrast, unsupplemented cystic fibrosis patients with pancreatic achylia showed 80% hemolysis on the average, a range of 17-96%. The stability of erythrocytes to peroxide challenge related to plasma α-tocopherol in a sigmoidal manner. Plasma α-tocopherol levels of <100 μg/100 ml were associated with >90% hemolysis, whereas samples with >500 μg/100 ml were almost completely resistant to oxidant damage. The survival of autologous ^{51}Cr-labeled red cells was determined in 19 vitamin E deficient patients and found to be significantly ($p<.005$) decreased to 22.4 days in comparison to control subjects who showed a mean ^{51}Cr-$t_{1/2}$ of 27.0 days. Erythrocyte survival was measured in 6 subjects before and after dietary tocopherol supplementation. While in the vitamin E deficient state this group showed a shortened RBC life span with a mean ^{51}Cr-$t_{1/2}$ of 19.1 days, whereas after supplementation the figure rose significantly ($p<.001$) to 27.6 days. Static hematologic indices, however, such as red cell count, hemaglobin concentration, and hematocrit value, were generally normal and did not rise consistently in patients supplemented with vitamin E. Thus, as the ^{51}Cr-RBC data suggest, the degree of hemolysis *in vivo* is minimal and not sufficient to cause frank hemolytic anemia.

Measure of urinary creatine excretion was carried out in an attempt to assess muscle function. Excessive creatinuria was observed in nearly all the 25 subjects so evaluated; however, this index did not consistently improve upon supplementation with vitamin E. Furthermore, only two of 40 vitamin E deficient subjects showed elevations in muscle-derived plasma enzymes, such as creatine kinase and aldolase. In addition, skeletal muscle samples obtained by biopsy from 5 subjects failed to reveal evidence of gross myopathy. Thus, the creatinuria is likely attributable to chronic wasting rather than acute or sub-acute muscular degeneration. Morphological studies on biopsy-procured jejunal mucosa revealed no evidence of ultrastructural changes. In particular, subcellular membranous organelles were preserved in vitamin E deficient patients.

It is concluded that vitamin E deficiency is regularly associated with cystic fibrosis providing such patients have pancreatic involvement and are not taking supplementary doses of the vitamin. Concomitant hematologic effects consistent with mild hemolysis, but not anemia, are found in these patients but may be reversed upon vitamin E supplementation.

Megavitamin E supplementation in man: To assess possible toxicity or beneficial effects of vitamin E, a group of 28 adults voluntarily ingesting 100 to 800 IU/day of vitamin E for an average of 3 yrs were evaluated in this study. Half of the subjects claimed a feeling of improved health or well-being, but no specific beneficial effects were noted consistently. Half of the subjects indicated no change in health status after beginning vitamin E supplements. No gross evidence of toxicity was apparent upon reviewing the past medical history with the subjects. Plasma α-tocopherol was found to be elevated significantly in the group, from 740 μg/100 ml (control mean) to 1340 μg/100 ml. However, 25% of the values were within 2 S.D. of the control mean, despite repeated claims that these subjects were taking the originally indicated dose. Plasma α-tocopherol levels did not correlate with total daily dose but did relate to plasma triglyceride and cholesterol concentrations. Total plasma carotenoids were increased significantly along with vitamin A levels; the former did not correlate with plasma vitamin E, whereas the latter showed a significant correlation. Laboratory screening for toxic side effects of the vitamin by performance of 22 standard clinical blood tests failed to reveal any disturbance in liver, kidney, muscle, thyroid gland, erythrocyte, leukocytes, coagulation parameters, or blood glucose. It is concluded that megavitamin E supplements in this group produced no apparent toxic side effects and that subjective claims for beneficial effects were highly variable.

Significance to Biomedical Research:

1) Findings thus far suggest that blood levels of polyamines are disturbed in cystic fibrosis homozygotes and heterozygotes. Further study will be required to determine if this apparent abnormality will be useful in population screening for carriers of the CF genotype. In addition, in the view of the widespread distribution of polyamines in cells and tissues as well as their probable abnormality in tissue culture fibroblasts, further investigations may provide an insight as to the nature of the elusive basic defect in cystic fibrosis. The polyamine abnormality is the first consistent abnormality of a low molecular substance which can be defined chemically and which is present in both homo- and heterozygotes for the CF gene.

2) In this project, a new assay for ciliary motility based on ATP hydrolysis, has been developed and for the first time a ciliary ATPase, similar to flagellar dynein, has been isolated and partially characterized. Measurement of ciliary ATPase activity and utilization of ATP by motile cilia in the presence of CF sera has revealed no differences as compared with assays in the presence of normal sera. Studies are continuing but this objective, quantitative assay has thus far failed to demonstrate the "CF factor."

3) These investigations which are still in course indicate so far that vitamin E deficiency in man is associated with many of the findings present in animals, but generally at a subclinical level. Such findings are: 1) Hemolysis, both *in vitro* and *in vivo* which can be corrected with administration of vitamin E or the addition of tocopherol to the *in vitro* system. 2) Evidence of muscle involvement as shown by creatinuria, occasional elevation of plasma activities of muscle derived enzymes, and deposition of ceroid pigment in smooth muscle.

The metabolic role of this ubiquitous biological antioxidant is still unclear, but its widespread presence in human tissues must serve a purpose. Patients with malabsorption and secondary tocopherol deficiency should, therefore, receive supplements of vitamin E. Such supplementation should also be considered in the dietary makeup of populations in areas of the world (e.g., Bangladesh, Jordan, etc.) suffering from malnutrition and receiving inadequate diets. However, there is little reason to add vitamin E supplements in countries such as the United States where people ingest adequate amounts of the vitamin in their diets and have normal blood tocopherol levels.

Keyword Descriptors: cystic fibrosis; polyamines; red blood cells; ciliary dyskinesia; vitamin E deficiency

Honors and awards: None

Publications: Lundgren, D.W., Farrell, P.M., and di Sant'Agnese, P.A.: Polyamine alterations in blood of male homozygotes and heterozygotes for cystic fibrosis. Clin. Chim. Acta, 1975 (in press).

Project No. Z01 AM 49001-02 PMB
1. Pediatric Metabolism
2.
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Clinical Studies in Cystic Fibrosis

Previous Serial No.: NIAMDD-PMB-2c

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., Lawrence F. Cohen, M.D., NIAMDD; Herbert Y. Reynolds, M.D., NIAID.

Cooperating Units: Laboratory of Clinical Investigation, NIAID.

Man Years: Total: 3.5
Professional: 2.0
Other: 1.5

Project Description:

1) Pseudomonas vaccine in cystic fibrosis: Colonization of the respiratory tract with Pseudomonas aeruginosa occurs in most patients with cystic fibrosis (80% of the patient population followed at the NIH). This contributes to the morbidity and mortality in CF by increasing bronchial obstruction and leading to bronchiectasis and necrotizing pneumonia. Pseudomonas organisms are notoriously resistant to most of the commonly used antibiotics, and therefore treatment may be difficult. Enhancement of the humoral and cellular pulmonary defense mechanisms of the patients may be another way of approaching the clinical problem of Pseudomonas infection in CF. We have employed a multivalent lipopolysaccharide antigen (Pseudogen, Parke-Davis Co.) to study the antibody responses of 12 CF patients. Each patient was given 6 doses of the vaccine intramuscularly over a two-week period. Although side effects did occur (fever, malaise, local tenderness, and induration) no patient had to be withdrawn from the study because of adverse reactions to the vaccine. Serum hemagglutinin titers to each of the seven serotypes contained in the vaccine were determined before and at intervals following the immunization. Good antibody response, i.e., significant antibody titer increases, were found in all patients. Elevated titers persisted for 3-6 months and then returned to baseline values.

In the small group of patients studied, no objective improvement in pulmonary function or clinical status was noted. Pseudomonas was not eradicated from any patient's sputum. Two patients had what they felt were significant, albeit subjective, improvements for several months following immunization. We conclude from our studies to date that although good antibody responses can be achieved without serious side effects, there is no significant clinical response in patients in whom Pseudomonas colonization is already well established. Clinical trials may be warranted in a larger number of patients, concentrating on those in whom Pseudomonas colonization has not yet occurred. (The intranasal route for immunization would seem well suited for this approach, if it is effective in producing significant antibody responses.)

2) Mucociliary velocity and transport in cystic fibrosis: Chronic pulmonary disease, secondary to bronchiolar and bronchial obstruction and bacterial infection, is the major cause of morbidity and mortality in cystic fibrosis. It has long been assumed that this was due to increased viscosity of pulmonary secretions with subsequent decreased mucociliary airway clearance. Recently *in vivo* studies of mucociliary airway clearance have suggested that clearance rates in CF patients were in fact faster than normal (New Eng. J. Med. 288: 651, 1973).

We have studied tracheal mucus velocity in patients with cystic fibrosis by a new technique (J. Appl. Physiol. 34:495, 1973). A fiberoptic bronchoscope is passed into the trachea of conscious volunteers under topical lidocaine anasthesia. Teflon discs are then blown onto the tracheal mucosa and their movement filmed with a movie camera. The discs are 0.67 mm in diameter and weigh 0.13 mg, well within the transport capabilities of the mucociliary system. The rate of movement is calculated from the change in image size of each disc with time, and is accurate within 10%. Xylocaine has been shown not to alter mucus velocity in dogs and sheep.

The average rate of mucus velocity in 14 patients with CF (ages 18-43, mean = 25) was 2.6 ± 3.3 mm/min (std. dev.). This was markedly slower than the rate in 20 normal volunteers (ages 18-44, mean = 27): 20.1 mm/min ± 6.4 (std. dev.) ($p<.001$). There was no co-relation of mucus velocity with age, sex, clinical status or pulmonary functions.

Following the measurement of mucus velocity, each subject was given 0.25 mg Terbutaline, a new beta-adrenergic agent; by subcutaneous injection. Fifteen min later the mucus velocity was measured again. There was no significant effect in normal volunteers, but in the CF patients the mucus velocity increased to 5.5 mm/min ± 3.6 mm/min ($p<.001$).

Significance to Biomedical Research:

1) Most patients with CF have chronic pulmonary colonization with Pseudomonas which is an organism notoriously resistant to most antibiotics. The development of an effective immunization program against Pseudomonas species could be of significant therapeutic benefit to these patients.

2) This is the first demonstration of pharmacological increases in mucociliary clearance in CF. While the achieved rates of mucus transport following the injection of Terbutaline are still only one-third of normal, we believe that further increases may be possible by different drugs, routes of administration, or drug combinations. This observation has great potential benefit in the therapy of patients with the chronic obstructive lung disease of CF.

Keyword Descriptors: cystic fibrosis; Pseudomonas aeruginosa; chronic pulmonary disease; tracheal mucus velocity

Honors and Awards: None

Publications: Pennington, J.E., Reynolds, H.Y., Wood, R.E., Robinson, R.A., and Levine, A.S.: Use of a Pseudomonas aeruginosa vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med. 58:629, 1975.

Project No. Z01 AM 49002-02 PMB
1. Pediatric Metabolism
2.
3. Bethesda, MD

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Pulmonary Lecithin Synthesis as Related to the Respiratory Distress Syndrome

Previous Serial No.: NIAMDD-PMB-3c

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., David W. Lundgren, Ph.D., NIAMDD: Ronald A. Chez, M.D., Michael F. Epstein, M.D., NICHD

Cooperating Units: Pregnancy Research Branch, NICHD

Man Years Total: 2.0
Professional: 1.0
Other: 1.0

Project Description:

Objectives: Considerable evidence has accumulated suggesting that atelectasis in RDS develops as a result of deficient pulmonary surfactant, particularly lecithin (Pediatrics 40:13, 1967). In our studies, biochemical regulation of lecithin synthesis has been evaluated in fetal lungs from various species. Recently it has been found that by obtaining samples of amniotic fluid of mothers during gestation and assessing the content of lecithin which rises rapidly in late pregnancy, it is possible to predict the patients who are going to be at risk of RDS at birth. This, of course, is especially so if a mother has previously had a child with RDS so that the subsequent ones are apt to have the same manifestations.

The compound is produced *de novo* by two pathways: the choline incorporation route (I) and the phosphatidyl ethanolamine methylation (II).

Our studies have demonstrated that pathway I is predominant and accounts for increased lecithin synthesis in the mature fetus as well as for the surge in amniotic fluid lecithin/sphingomyelin ratio.

Enzymatic synthesis of phosphatidylcholine (PC) in homogeneous cultures of apparent type II pneumonocytes: Accelerated production of PC-rich pulmonary surfactant occurs following treatment with various hormones. To delineate pathways and regulation of PC biosynthesis, investigations are being conducted with diploid epithelial cells isolated from rat lung (clone L-2) which have been shown to contain osmiophilic lamellar (*In Vitro* 10, 1975). Studies to date have employed over 50 cultures of L-2 cells grown in either a basal medium (control) or in the presence of hormones such as thyroxin and cortisol. The three catalysts of the principal *de novo* route for PC synthesis - the choline pathway - were readily detected in control cells and compared in terms of specific enzymatic activities with rat lung homogenates and fibroblasts from lung and skin. Results indicate that L-2 cells are enriched to as high as tenfold in choline kinase (CK), cholinephosphate cytidyl transferase, and cholinephosphotransferase (CPT). Cells continuously cultured in the presence of thyroxin showed markedly increased CK (mean±SE = 4815±353 cpm product/min/mg protein; control = 2858±232) but reduced CPT (both $p<.001$). Although cortisol alone produced only slight enzyme elevations, its addition to thyroxin-supplemented cells led to a seven-fold increase in CPT activity ($p<.001$).

It may be concluded that L-2 cells in tissue culture are 1) capable of active PC production, and 2) respond to hormones in a manner consistent with observations on lungs of treated animals.

Correlation of fetal lecithin synthesis and the amniotic fluid (AF) L/S ratio in primates: Rhesus monkey gestations were utilized to examine the relationship between the AF L/S ratio and fetal lung lecithin synthesis. In 24 fetuses (114-161 days, term 164) the rate of lung lecithin synthesis *in vitro* via choline incorporation (I) and phosphatidylethanolamine (PE) methylation (II) was determined by incubating lung slices with 2 μCi of ^{14}C-choline or ^{14}C-methionine. Choline incorporation accounted for 90% of lung lecithin synthesis in all fetuses. Pathway I activity was stable from 114-148 days and then abruptly increased with peak activity (3 x baseline, $p<.001$) at day 150 (90% term). The AF L/S ratio, determined in these and 60 additional gestations (70-161 days) showed a gestational pattern similar to that in humans with an abrupt increase at 90% of term. The correlation between lung choline incorporation and AF L/S ratio in the last 30% of gestation was significant ($r = .77$, $p<.001$); lung PE methylation showed no correlation with the L/S ratio. To confirm that lung choline incorporation into lecithin determines the AF L/S ratio, 1 mCi of ^{14}C-choline was injected into the maternal aorta (2 monkeys, 142 days gestation) to provide labelled precursor for fetal lung ^{14}C-lecithin synthesis. AF ^{14}C-lecithin specific activity was determined every 48 hrs over 2 weeks; it increased gradually until day 150 when an abrupt 2.5 fold increase ($5.6 \rightarrow 12$ & $2.8 \rightarrow 7.4$ cpm/μg lecithin) occurred. This was coincident with a 30% and 120% increase in the L/S ratio ($3.4 \rightarrow 4.4$ & $2.3 \rightarrow 5.1$). It is concluded that the AF L/S ratio accurately reflects developmental changes in fetal lung lecithin synthesis.

Primate fetal lung in gestations complicated by maternal glucose intolerance: The effects of maternal glucose intolerance on primate fetal lung lecithin synthesis were studied in the Macaca mulatta. Ten monkeys in the first trimester were treated with streptozotocin (Stz, 47 mg/kg IV), a pancreatic beta-cell cytotoxin, with resultant impairment of maternal insulin secretion and glucose clearance in response to IV GTT's. Ten pregnancies, delivered by C-section at 86-89% term, showed changes similar to those seen in human diabetic pregnancies - fetal macrosomia and beta-cell hyperplasia, hydramnios, and placental hyperplasia. *De novo* pulmonary lecithin synthesis via the two major pathways - choline incorporation (I) and phosphatidylethanolamine methylation (II) was measured in fetal lung slices incubated with 2 μCi of ^{14}C-choline or ^{14}C-methionine and the amniotic fluid (AF) L/S ratio was determined. Five fetuses of normal, age-matched gestations were controls.

Results:	Pathway I	Pathway II	AF L/S Ratio
Stz	800 ± 90*	90 ± 35	2.5 ± 0.4*
Normal	490 ± 60	20 ± 1	1.0 ± 0.2

Pathway I activity (cpm/mg lung/hr) accounted for 90-96% of lecithin synthesis in both groups. Pathway I activity and AF L/S ratio were significantly (*p<.05) greater in the Stz group; pathway II was not. Therefore, maternal glucose intolerance was associated with an increase in fetal lung choline incorporation into lecithin, reflected in an earlier increase in the AF L/S ratio. This suggests that in the human it may be the timing and mode of delivery rather than maternal diabetes mellitus that results in the increased risk of RDS in the neonate.

Keyword Descriptors: respiratory distress syndrome; pulmonary lecithin synthesis;

Honors and Awards: None

CLINICAL HEMATOLOGY BRANCH, NIAMDD

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis
Dr. N. Raphael Shulman

1. Identification of Intermediates in the Pathway of Prothrombin Conversion.

Following our previous demonstration in an impure biological system that prothrombin forms at least one intermediate derivative before thrombin, we have attempted to further define the nature of prothombin intermediates by converting prothrombin to thrombin in a highly purified system. Rather than impure biological activators we have used highly purified Factor X and Factor V as the converting agents and highly pure prothrombin as the substrate. In this new system it is apparent that at least two and possibly three derivatives of prothrombin form prior to formation of thrombin. These derivatives and the fragments of prothrombin cleaved by activators were identified primarily by acrylamide gel electrophoresis combined with kinetic analyses of the conversion system. Of special interest was the finding that soy bean trypsin inhibitor prevented derivative formation during the earliest stages of prothrombin conversion. Although kinetic analyses of the inhibitory reaction suggests that soy bean trypsin inhibitor competes with conversion factors in a rate-dependent manner to form a derivative-soy complex, separation of this complex from prothrombin and its other derivatives has been elusive because electrophoresis leads to separation of the complex. Currently we are using a variety of affinity gels in attempts to purify the derivative that combines with soy bean inhibitor. Because there are proteolytic inhibitors in plasma with anticoagulant properties similar to that of soy bean inhibitor, the reaction may be important in homeostatic mechanisms that prevent thrombosis. Current work suggests that the rate-dependence of the inhibitory reaction would effectively block slow rates of prothrombin conversion that might be expected to occur intravascularly but not interfere with rapid rates of prothrombin conversion stimulated by tissue factors when blood is extravasated by vascular trauma.

2. Molecular Changes in Factor VIII (F. VIII) During Clotting.

Following our previous findings that F. VIII could be separated from plasma by gel filtration on Sepharose 4B following limited digestion of plasma by chymotrypsin and the demonstration by others that high ionic strength produced fragmentation of F. VIII, we studied the effects of the clotting process on the molecular and functional characteristics of F. VIII. These studies combined with those of last year, indicate that during the clotting process F. VIII separates into at least two components, one with a molecular weight (MW) of approximately 2,000,000 and the other in the range of 100,000-200,000. The high MW component is inert in the clotting process but neutralizes antibodies directed against F. VIII. The low MW component is highly active as a procoagulant and also neutralizes anti-F. VIII antibodies. These two components are found in fresh normal serum whereas plasma contains only a high MW species of F. VIII. Since thrombin treatment of plasma results in formation of low MW F. VIII it is possible that thrombin is responsible for dissociation of the F. VIII molecule and perhaps thereby for the activation of F. VIII in the clotting sequence. The mechanism of activation of F. VIII heretofore has been obscure.

Studies of the high and low MW components in plasma and serum of patients with classical hemophilia or Von Willebrand's disease shed some light on the molecular abnormalities of these disorders. Hemophilic plasma contains an inert high MW F. VIII component but no evidence was found for the physical existence of the low MW component after thrombin treatment of hemophilic plasma. On the other hand, Von Willebrand's disease plasma, when treated with thrombin, contains both high and MW components but on the basis of reactions with anti-F. VIII, there appears to be a quantitative and/or qualitative deficiency in the small component despite its procoagulant acitivity.

3. Studies of the Congenital Platelet Abnormality, Thrombasthenia.

Physiological and therapeutic studies were carried out on two patients with Glanzmann's thrombasthenia, a congenital hemorrhagic disorder associated with normal numbers of platelets, but a prolonged bleeding time and impairment of clot retraction. There was no evidence of a metabolic disorder as judged by glucose oxydation, lactate production and incorporation of acetate into lipids by the platelets of these patients. The ability of platelets to release intracellular granules under the stimulus of APD , collagen or thrombin was intact. However, subsequent to the release reaction, thrombasthenic platelets did not aggregate, as do normal platelets. Degree of aggregation in mixtures of normal and thrombasthenic platelets was directly proportional to the fraction of normal platelets in the mixture indicating no interaction between normal and thrombasthenic platelets. Normal plasma did not correct the thrombasthenic abnormalities nor did plasma from thrombasthenic patients impair normal platelet function. The clot retraction abnormality was not corrected even in mixtures containing 75% normal platelets. In keeping with *in vitro* observations, infusion of normal platelets sufficient to double the patient's platelet count, provided only partial transient correction of the bleeding time in one patient and gave no improvement at all in the other patient. *In vivo* inhibition of normal platelet function by thrombasthenic platelets correlated best with *in vitro* inhibition of clot retraction. This observation accounts for the failure of thrombasthenic patients to respond to plasma, whole blood, or platelet transfusions that have been tried in the past. It appeared from these studies that effective therapy in thrombasthenia would require removal of abnormal platelets by plasmaphoresis prior to transfusion of normal platelets.

II. Study of Immunology of Blood Cell Deficiences (N. R. Shulman)

1. An Immunologic Test for Fibrin and Fibrinogen Degradation Products.

Thrombocytopenia secondary to intravascular clotting is a common clinical occurence that is difficult to diagnose because of limitations in assay systems currently employed for measuring serum fibrin or fibrinogen degradation products. At present there are several assays in use; notably, the tanned red cell hemagglutination immunoassay, flocculation tests, immunodiffusion, and protamine sulfate or ethanol gelation tests. Each of these systems has certain limitations relative to sensitivity or specificity. In this work we sought to develop a simple, rapid and sensitive technique for measuring degradation products of fibrinogen and fibrin based on an immunologic technique involving counterelectrophoresis of the test serum against antifibrinogen antibodies. In this test a precipitin line forms between opposing wells of an agar plate at concentrations of antigen (split products) that are clinically significant but does not form with normal serum that contains split products in concentrations too low to develop a precipitin agglutination line. The intensity of the line is proportional to the severity of intravascular clotting and the number of lines that form can be clearly related to the concomitant process of fibrinolysis, which takes place as result of intravascular clotting. This test has been run in tandem with other tests currently available and seems to provide a better, simpler and more rapid estimate of intravascular clotting than do others.

2. Malaria Thrombocytopenia

It has been known for several years that thrombocytopenia is a consistant occurence in early malaria. In attempting to determine the cause of thrombocytopenia in this disorder we studied Plasmodium cynomolgi infection in Rhesus monkeys and P. vivax malaria in man, the physiologic manifestations of both being almost identical. A concept entrenched in the literature concerning thrombocytopenia of malaria suggests that it results from intravascular clotting, secondary to hemolysis with utilization of platelets in the clotting process. We found no evidence for intravascular clotting by performing quantitative assays of all identifiable clotting factors and at no time during the course of either human or simian malaria were coagulation or fibrinolytic abnormalities found. There was no evidence of decreased production of platelets by a variety of techniques and no antiplatelet factor circulating during the thrombocytopenic phase as judged by transfer of plasma to normal animals. However, it was found that anti-malaria antibodies began rising and complement levels falling sharply at the time thrombocytopenia developed. Of the various possible mechanisms accounting for thrombocytopenia, it appears likely that immune complexes of malaria antigen with antibody may be adsorbed on cells and lead to their sequestration as in other forms of immunologic thrombocytopenia, elicited for example by drugs or certain isoantigens.

3. Relationships Between Platelet Function and Cyclic Nucleotide Metabolism.

In previous studies we found that agents which cause platelet aggregation and release of platelet granules markedly decrease adenylate cyclase activity but do not significantly change cyclic AMP levels of platelets sonically disrupted after aggregation. In order to determine whether the decreased adenylate cyclase was an artifact of an impure system, we determined the recovery of a number of soluble and membrane bound enzymes in platelets. The recovery of adenylate cyclase was comparable to the recovery of membrane bound platelet, collagen: glucosyltransferase and the recovery of cyclic nucleotide phosphodiesterase in the soluble fraction was similar to recovery of the known soluble cytoplasmic enzyme, lactic dehydrogenase. Because of the controversy in the literature concerning validity of observations on adenylate cyclase, we feel that these control studies were necessary to adequately substantiate the validity of changes in adenylate cyclase activity associated with the platelet aggregation and release reactions. Current work concerning platelet nucleotide metabolism concerns changes in guanylate cyclase and cyclic GMP during aggregation and release. It appears that most reagents responsible for the release reaction increase guanylate cyclase activity in contrast to the decrease in adenylate cyclase activity. Of special interest has been attempt, so far nonconclusive, to see whether immunologic reactions that affect platelets cause the same changes in cyclic nucleotide metabolism that other aggregating agents do.

Project No. Z01 AM 51,000-18-CHB
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Immunology of Blood Cell Deficiencies

Previous Serial Number: NIAMDD-CHB-2c

Principal Investigator: Dr. N. Raphael Shulman

Person Years:

Total:	1 1/2
Professional	1/2
Other:	1

Project Description:

Objectives:

To study the nature of immunolgoic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homotransplantation. Immunologic techniques have been used to develop a number of clinically practical diagnostic tests for certain infectious diseases and drug addiction.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physicochemical characterization of purified antibodies and antigens, microanalyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutination and precipitin reactions, immunoelectrophoresis, methods of provoking antibody responses in man and animals, and isotopic and flourescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination and techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocytes by chemical coupling.

Keyword Descriptors:

1. autoimmunity, drug antibodies, platelet antigens, isoimmunization, transplantation antigens, hepatitis, hemagglutination tests, thrombocytopenia.

Publications:

1. Shulman, N. R.: Analyses of Drug Antibodies with Autoimmune Implications. Laboratory Diagnosis of Immunolgic Disorders. 1975.

Project No. Z01 AM 51,001-18-CHB
1. Clinical Hematology Branch
2.
3. Bethesda Maryland

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Previous Serial Number: NIAMDD:CHB-2c

Principal Investigators: Dr. N. Raphael Shulman

Other Investigators: None

Person Years
Total: 1 1/2
Professional 1/2
Other: 1

Project Description:

Objectives:

Study of the reactions and interactions of coagulation factors *in vitro* and *in vivo* to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactivity labeled substrates.

Keyword Descriptors:

1. Hemophilia, coagulation mechanism, clotting factor assays, clotting factor purification, platelet physiology, platelet metabolism.

Publications:

1. Shulman, N. R., and Gordon, N. R.: The Effectof Clotting on Structure and Function of Human Factor VIII. Annals of the New York Academy of Sciences. 240:79, 1975.

2. Sarna, G., Tomasulo, P., Lotz, M. J., Bubinak, J., and Shulman, N. R. Multiple Neoplasms in Two Siblings with a Variant Form of Franconi's Anemia. Cancer, in press.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
DIGESTIVE DISEASES AND NUTRITION

ASSOCIATE DIRECTOR'S REPORT

The new Associate Director for Digestive Diseases and Nutrition, Dr. Harold P. Roth, joined the cluster November 24, 1974. An initial period has been, to an important extent, involved in becoming acquainted with the current activities of the cluster and the technical processes involved in NIH activity, and the beginning of an assessment of activities in digestive diseases in other Institutes. Other activities include the following:

A survey of opportunities and needs in research on digestive diseases was begun two years ago. This study involved many scientists from the gastrointestinal community, who identified the areas that were important and promising in the fields of their expertise. A report has been assembled and edited, and it is being prepared for publication in Gastroenterology in the Fall. A less technical report has also been prepared for distribution to interested laymen. It is important to continue with regular assessments of the potentially fruitful areas for research with this document as a background. Accordingly, a committee was appointed to select appropriate scientists who would assemble at regular intervals to discuss the field and make recommendations. The committee will also consider means of implementing their suggestions. They will coordinate their program with the activities of scientific societies in the area of digestive diseases and nutrition. They may also recommend workshops--and appropriate publications such as state of the art reviews or editorials. This committee's activities should be of help to the NIH in its programming.

A report on risks in human experimentation was also recently completed. The responsible committee was partially sponsored through the NIH. It is planned that the activities initiated by this group be furthered through the activities of specific committees from the relevant scientific societies. For example, risks in endoscopy will be considered by committees from the American Endoscopic Association. Guidance in evaluating the risk of procedures could then be obtained through appointed consultants identified for their expertise by the specific societies. One finding of the committee was the lack of adequate data derived from prospective studies on the risks of various procedures. It is hoped that appropriate workshops and discussions will lead to the development of plans to obtain the necessary prospective data. Some societies have already indicated their willingness to become involved in this type of activity. For example, at a meeting partially sponsored by the NIH, the risks of cannulation of the common bile duct and the pancreatic ducts were reviewed, and plans for prospective studies were discussed.

A program to provide special materials to aid in research was initiated. The value of uniform high quality preparations of gastrointestinal hormones to facilitate good research in gastrointestinal endocrinology was recognized. A committee was organized to guide and advise the NIH in proceeding in this area. Plans have been made to supply a minimal amount of hormone material initially. Under the committee's guidance, the value of expanding this activity will be explored. The possibility that other biological materials should be made available is also being examined.

The digestive diseases program has been divided and a specific program in liver disease has been established. This is consistent with the growing activity in the field of liver disease, the establishment of separate training programs in the area, and the growing body of scientists who specialize primarily in the area. This program has been taken over by Dr. Sarah Kalser as program director and it will include liver and biliary tract disease.

The Digestive Diseases Program encompasses research of organ systems associated with the gastrointestinal tract. Research activity is concerned with the basic aspects of the physiology and biochemistry of disease entities. Efforts include studies of the cause, prevention, diagnosis and treatment of diseases of the esophagus, stomach and duodenum, pancreas and salivary glands; inflammatory and metabolic diseases of the liver; diseases of the gallbladder and bile passages; absorptive and secretory diseases of the small and large bowel; colonic and inflammatory bowel diseases; gastrointestinal circulatory disease. Also included in this program area are studies related to the psychosocial aspects of digestive diseases; and research, development and evaluation of techniques, methods, or approaches in the use of endoscopic and other instrumentation for the diagnosis, treatment, and investigation of digestive diseases.

A. Research Grants

During July 1, 1974 through June 30, 1975 approximately 257 research grants, 2 centers and 3 program projects, 32 training programs, 27 career awards and 19 fellowships have been supported in the Digestive Diseases Program area.

Of the many significant advances made in the Digestive Diseases area during the past year, by Institute-supported grantees, only a few examples will be cited. These deal with the following disorders:

1. Crohn's Disease
2. Duodenal Ulcer
3. Absorption of Antigens
4. Hepatobiliary Disease
5. Gallstones

1. Crohn's Disease

With additional confirmatory testing, a very valuable diagnostic tool may be finally available with which to evaluate the activity of a severe disease of the small intestine, Crohn's disease. This disease, in which the terminal part of the ileum is usually involved and which may lead to intestinal obstruction, fistula formation and growth retardation, is often difficult to distinguish clinically from ulcerative colitis, also an inflammatory disease but of the colon. Now some grantees at the Massachusetts General Hospital have found that a serum enzyme, lysozyme, is increased in Crohn's disease. Moreover, there appears to be relatively good correlation between the level of enzyme and the activitiy of the disease. Thus, of the 54 patients with Crohn's disease evaluated, 46 patients with mild to severe disease as assessed by clinical, laboratory and radiological findings, also showed significantly increased serum lysozyme concentration. In the 8 patients in which the disease was inactive, the serum lysozyme concentration was normal. However, there were some patients with mild to moderately active disease in which the serum level was normal or only slightly elevated. Thus the test needs further refinement, but it does seem to offer promise as a means of monitoring the course and response to treatment of a disease, otherwise difficult to assess.

2. Duodenal Ulcer

A potent compound to inhibit gastric acid secretion in patients with duodenal ulcers has been examined by grantees at the Center for Ulcer Research and Education at the University of California in Los Angeles. When compared with the conventional antisecretory compound, probanthine at its maximally effective dose, the prostaglandin E_2 compound produced a much more profound (essentially complete) suppression of the gastric acid secretion in response to a meal. It was able to exert this effect, when administered orally, for at least 2 hours and at a dose small enough to prevent changes in pulse or blood pressure. It may act by inhibiting gastrin release. Additional evaluation of prostaglandin E_2 is certainly indicated, as it may be of therapeutic benefit to duodenal ulcer patients.

A group of investigators at the University of Texas, Southwestern Medical School, are working on the gastric secretion problem from another point of attack, the histamine receptor. They have tested an analogue of an effective blocking agent, metiamide, which had to be abandonned because of certain adverse effects. Preliminary studies indicate that this new compound, cimetidine, is just as effective and has a longer duration of action than metiamide. Whether it, too, will have adverse side effects has not yet been clearly established.

3. Absorption of Antigens

The role of the exocrine pancreatic secretion, with its complement of enzymes for the digestion of starches, fats and proteins, may also be important in protecting the body from antigenic macromolecules. Grantees at Harvard Medical School and Massachusetts General Hospital have examined the mechanism by which immunization interferes with the intestinal absorption of macromolecules. Formation of an antigen-antibody complex on the surface of the gut not only seems to prevent the binding of additional antigen to gut cells, but it also speeds up the degradation by gut proteases of the antigen in the complex. These proteases seem to be of pancreatic origin because when the pancreatic fluid is removed from the intestine of immunized rats, the break-down of antigen is significantly slower than when pancreatic juice is present. This may be an important function of the pancreas; i.e. to limit the absorption of ingested antigen and thus prevent the deleterious systemic consequences of antigenic and toxigenic macromolecules.

4. Hepatobiliary Disease

Although many tests to evaluate liver function are currently available, there is still the need for analytical procedures of greater sensitivity and specificity. The radioimmunoassay described by grantees at the Mayo Foundation and the Pennsylvania State University for assaying four of the serum bile acids looks promising, although additional work is needed for validation. Differing patterns of evaluation of serum bile acids were seen in patients with cirrhosis, hepatitis, cholectasis and liver cancer.

5. Gallstones

Unconjugated bilirubin, a normal component of bile, has a markedly decreased solubility as the pH of the bile drops. Since a drop in pH occurs in the stagnant gallbladder, a new hypothesis is being offered as a possible mechanism for gallstone formation by grantees at the University of Pennsylvania; i.e. that the low pH may cause unconjugated bilirubin to precipitate from bile and thus trigger gallstone formation. Obviously, since this information was collected from in vitro systems work needs to be performed in vivo to verify or refute this hypothesis.

B. Contracts

1. Two ongoing contract efforts have proceeded according to schedule.

   a. Crohn's Disease Cooperative Study

   Fourteen treatment centers have accessioned 507 patients into the two phases of the study, the 4 month treatment phase and the 24 month prophylactic phase. Since this randomized, double-blind study is still in progress, the data are not yet ready for analysis, although significant trends have already been noted.

b. National Cooperative Gallstone Study

The Coordinating Center with its committees has completed a protocol, procedure manual and medical forms and has obtained approval of the Food and Drug Administration to start the 1000 patient two-year clinical trial. The purpose of the trial is to evaluate the safety and efficacy of two different doses of chenodeoxycholic acid on the dissolution of gallstones. The entire effort is now undergoing final NIH review before the actual trial is begun.

2. New contract efforts in the area of endoscopic research are currently undergoing final evaluation and negotiation. These studies were selected from 49 proposals received for review. It is anticipated that three contracts will be awarded to study the control of gastrointestinal bleeding by endoscopic systems involving laser coagulation, electrocoagulation, suture clips, and tissue glues. Also, a minor project will be supported to improve techniques of canulation of the biliary and pancreatic ducts in conjunction with endoscopic retrograde cholangiopancreatography (ERCP). ERCP is currently regarded as a valuable adjunct to the diagnosis of pancreatic and biliary disease.

3. To partially satisfy the urgent requirement for supplies of gastrointestinal hormones for research programs, it is anticipated that a contract will be awarded to Dr. Viktor Mutt of the Karolinska Institute in Sweden to obtain small quantities of three purified hormones, namely, secretin, cholecystokinin, (CCK) and vasoactive intestinal peptide (VIP). Plans are also being formulated to expand this effort to obtain these and other difficult-to-obtain gastrointestinal hormones on a sustained basis. These will be distributed to qualified investigators upon request and selection by an appropriate review mechanism.

Nutrition Program Area

This Nutrition Program of NIAMDD-EP continues to support research and training in basic and clinical nutrition directed toward improvement of human health and longevity. Other extramural areas of NIAMDD (notably Diabetes, Metabolism, Endocrinology and Hematology) support research involving Nutrition as a secondary but significant component. Generallly speaking, NIAMDD supports approximately 40 per cent of nutrition research represented in the different Institutes of NIH, and carries a proportionately greater responsibility for training through fellowships and training grants than other Institutes combined.

Trends and Developments in Supported Research

1. Obesity In last years report it was noted that while efforts to establish Centers for Obesity Research had not been as successful as hoped for, support for research on factors regulating appetite, feeding behavior, food intake, weight regulation and obesity *per se* was reasonably good. As compared to a total of 19 research projects primarily related to obesity in Fiscal '74, there have been funded 12 additional projects of high scientific merit during Fiscal '75. Undoubtedly, much of this expansion reflects stimulus given through programming efforts related to the proposed establishment of Obesity Centers, and might well accomplish as much or more than the original hope or intent.

2. Parenteral Therapy Second only to obesity, it has been considered that total parenteral nutrition, and many unanswered questions concerning the nutritional components of parenteral fluids (especially the development of a safe type of lipid necessary for the prevention of essential fatty acid deficiency) should have high priority in future research support of NIAMDD. This need is imperative for modern medicine to properly utilize total parenteral nutrition in the proper management of congenital anomalies and various disorders of the gastrointestinal tract, renal and hepatic failure, trauma, burns and post-surgical states. Some programming has been initiated to augment the rather miniscule support being given in this important area, and there are encouraging prospects that the three research proposals relevant to this area may in Fiscal '76, be added to the three grants currently active.

3. Trace Elements Supported research has made some interesting advances in regard to the role of chromium, zinc selenium and silicon in biological processes. Two new research projects have been added to the 14 previously supported. It is anticipated that during the coming year there will be a significant advance in identification of the chromium containing glucose tolerance factor and in better understanding of its role in diabetes mellitus. New findings indicate an important role of silicon in osteoblast functions and bone formation.

4. **Proteins and Amino Acids** Research in areas pertaining to proteins, amino acids and lipids in nutrition has made steady progress. One area of increasing interest relates to the effectiveness of α - keto analogues of essential amino acids in the treatment of hyperammonemia, uremia and chronic renal failure. Because of the recognized value of these special amino acids in parenteral therapy for certain clinical disorders, and because of the limited supply available, consideration is being given to use of contract mechanisms to provide a more adequate supply for biomedical research and therapeutic uses.

5. **Vitamins** Important advances are being made in knowledge of the mechanisms of action of the vitamins - notably, the mode of action of metabolites and analogues of vitamin D, the role of the liver in secretion and release of retinol-binding-protein, and interrelationships of folic acid and vitamin B_{12}. NIAMDD is co-sponsoring a workshop conference on folic acid at the National Academy of Sciences on June 5, 1975.

6. **Dietary Fiber** There has recently been provided substantial evidence that increased consumption of highly refined cereal grains and other foods, with resultant decrease in dietary fiber, may lay the basis for certain gastrointestinal diseases (notably diverticulosis) and ischemic heart disease. It is anticipated that during the years to come there will be a rapidly increased focus of research pertaining to the role of dietary fiber in human nutrition. Consideration is being given by the staff of Digestive Diseases and Nutrition, to organization of a workshop on this subject during FY '76.

Malnutrition Panel (U.S.-Japan CMSP)

This Panel, under the new chairmanship of Dr. David Coursin (replacing Dr. Nevin Scrimshaw) continues its review of potential R22 research grants and contracts for relevance to its program priorities, and is in course of developing a series of position papers and workshops directed toward sharpening focus of research activity in the areas of its four priorities. Meanwhile, support at an approximate 1 M level has been given to 4 new and 7 renewal applications, which is comparable to the level of support for the same number of grants during FY '74.

Support for Research Projects (R01 and R22) Grants

Non-competing research grants funded during Fiscal '75 numbered 90 as compared to 93, 81 and 75 for the three previous years. Of the 78 grant applications (AM primary) approved by the past three Councils, 46 (or 59%) were funded. These figures do not include 4 applications approved prior to June 1974 with begin dates in FY '75. Of the total of 50 grants funded, 23 were new and 27 were competing renewals. This reflects a healthy introduction of new research projects into the program.

Fellowship, Career and Training

During FY '75 fellowship and training awards were active under a mixture of program authorities: the old postdoctoral (F02), special fellowship (F03) and training (T01) programs; the "Weinberger" individual postdoctoral research fellowship (F22); the National Research Service Awards for Individual Postdoctoral Fellows (F32) and Institutional Grants, NRSA for Research Training (T32).

In the closing hours of Fiscal '74 support was given to 15 applicants for F22 type fellowships. During Fiscal '75 two new Research Career Development Awards (K04), six new NRSA Individual Fellowships (F32) and one new NRSA Institutional Grant (T32) have been funded. One training grant which was eligible for released FY '73 impounded funds was awarded as a new T01, and one new type (T32) training grant, recently reviewed, is being funded. Seven of the original training grants (T01) are being phased out. During Fiscal '75 these grants supported 12 predoctoral and 13 postdoctoral trainees.

Official Duties

The Nutrition Program Director has continued to serve as Program Officer of the Malnutrition Panel of the U.S.-Japan Cooperative Medical Science Program, and as a member of the Food and Nutrition Advisory Council of the Nutrition Foundation, Inc. He has resigned as a member of the Scientific Advisory Committee of the Muscular Dystrophy Association, Inc., but has agreed to serve as a consultant to the Association and as a member of its Corporation.

ARTHRITIS PROGRAM AREA

The Arthritis Program encompasses research activities in a wide range of related subjects such as connective tissue (collagen) diseases, joints and their contents, inflammation, autoimmune reactions (immunology), and chemotherapy relative to arthritis.

Three major forms of arthritis exist: (1) rheumatoid arthritis, the most crippling form, (2) osteoarthritis, which is associated with old age and degeneration of joints, and (3) gout, a hereditary disease which predominates in males.

The most important and far-reaching event which occurred this fiscal year for arthritis research was the enactment into law on January 4, 1975 of the National Arthritis Act of 1974. This Act provides for the establishment of a National Arthritis Commission of 18 members to formulate a long-range plan with specific recommendations to utilize and organize national resources to combat arthritis. The Commission held its organizational meeting on April 7, 1975.

The Arthritis Program currently has 152 active research grants, 11 program projects, 24 training grants, 15 career development awards, 1 career award, and 23 fellowships. The number of active research grants in the last year and a half has increased somewhat due to release of impounded funds in FY 73 and to an increased budget in FY 75. Training grant support has remained almost constant in the last year even though the program is being phased out. Most of the current training grants will terminate next year. New training grant applications are being reviewed.

Collagen. The enzyme collagenase has long been thought to play an important role in collagen destruction. Recently a grantee has demonstrated that under normal conditions collagen fibers are resistant to collagenase. However, at temperatures of inflamed rheumatoid knee joints, the collagen breakdown was four times greater than in normal knee joints. Since treating inflamed knee joints with heat usually aggravates the condition, this finding suggests that treatment of arthritic joints with anti-inflammatory agents may lower the temperature inside the inflamed joint thus reducing the rate of collagen destruction.

Another grantee has thrown some light on the role of hydroxyproline in triple helix formation. Unhydroxylated collagen and procollagen has decreased thermal stability relative to the hydroxylated form. Proylhydroxylase can hydroxylate unhydroxylated collagen and facilitate triple helix formation during hydroxylation. The molecules containing proline and lysine analogs are not triple helical and thus less heat stable.

Another grantee has discovered that cartilage from osteoarthritic human femoral heads contains an inhibitor which is non-dialyzable and appears to be covalently bound to collagenase. Normal articular cartilage showed neither inhibitor nor enzyme.

Hormones and Endocrinology. Prostaglandins are hormone-like substances which act locally in many body tissues. A grantee has been investigating the relationship of prostaglandins with inflammation, usually the first manifestation of arthritis. This group has recently discovered that pharmacologic doses of prostaglandins are anti-inflammatory in nature in experimentally induced arthritis in rats. More recent experiments show that this effect is not mediated entirely by stimulation of the pituitary and/or adrenal gland and that other mechanisms are involved.

This group has also discovered that whole bee venom prevents experimentally induced arthritis in rats but the individual major fractions of bee venom do not. Whole bee venom produced a sustained elevation of serum corticosterone in rats but similar treatment by major components does not. Since prostaglandin E protects adrenalectomized rats against polyarthritis, it is theorized that the effects of prostaglandin E and bee venom on experimental arthritis are not necessarily related. Additional work in this field continues.

The data of another grantee suggest that normal articular cartilage may contain an inhibitor of prostaglandins derived from rheumatoid synovium. This inhibitor may be responsible for activation of lysosomes.

Still another grantee in measuring prostaglandin B in human synovial tissue discovered increased levels in untreated patients with inflammatory rheumatic diseases. These data suggest that increased prostaglandin levels may stimulate the inflammatory reaction and produce resorption of juxta-articular bone.

Synovial Membranes and Fluid. Recently one of our grantees has shown that enzymatically generated superoxide radical rapidly degrades bovine synovial fluid and solutions of purified hyaluronic acid. Degeneration was manifested by marked reduction in viscosity and an inability of degraded synovial fluid to form a "mucin clot" characteristic of healthy synovial fluid. Addition of catalase of SOD (superoxide dismutase) to synovial fluid provides protection against degradation. The superoxide production appears to be bactericidal in nature. Deterioration of synovial fluid is a symptom which characterizes inflammatory types of arthritis. Since injected SOD protects against this degradation, further study is taking place to determine if this phenomenon could be useful in arthritis prevention or control.

An NIAMDD-supported investigator has demonstrated differences in normal and rheumatoid synovial cells in tissue culture due partially to an "activator" peptide present in excess in rheumatoid cells. Characteristics of this peptide qualify it for a role as a key regulator of inflammation. Measurements show an early peak of activity and a continued excess of this "activator" peptide. A continued excess of it was shown to produce changes characteristic of chronic rheumatoid inflammation.

Immunology. B-microglobulin, usually present in low concentrations in serum and other body fluids, was found in elevated amounts in saliva and synovial fluid in patients with rheumatoid arthritis, and other autoimmune inflammatory diseases. These levels decreased in patients showing a clinical response to systemic treatment. This discovery may offer a simple method of quantifying local activity to autoimmune states.

A grantee has discovered that human neutrophils cause a release of degrading protease during cell contact with immobilized immunologic reagents and certain anti-arthritic drugs can inhibit the immunologic release of this neutral protease.

Macrophages (WBC's) incubated with lymphokines, products secreted by stimulated spleen cells, produce collagenase. These lymphokines were obtained from both mitogen and antigen-stimulated lymphocytes. These observations of a grantee suggest that the degradation of collagen in chronic inflammatory lesions may be caused by macrophage collagenase.

Gout. The discovery by a grantee that one type of gout is linked to an enzyme defect may lead to a new approach in combating this disease. An overactive enzyme is involved in the synthesis of a sugar phosphate, phosphoribosyl-l-pyro-phosphate from the substrate adenosine triphosphate and ribose-5-phosphate. This compound plays a key role in the formation of purine nucleotides. In the course of clinical studies this grantee discovered a family with the enzyme defect. Studies with them suggest that this is a most unusual type of genetic abnormality since it appears to be dominantly inherited. This finding further substantiates and strengthens evidence for a central role of PD-ribose-P in controlling the rate of purine synthesis. The fact that this type of gout is rare gives added support to the idea that a wide variety of primary genetic abnormalities are responsible for hyperuricemia and gouty arthritis.

Drugs. A grantee has demonstrated that patients with rheumatoid arthritis have a significantly decreased concentration of free histidine in their blood. Subsequent studies have demonstrated that a marked improvement occurs in patients treated with l-histidine.

Still another investigator has shown that adding copper to aspirin makes it a more effective anti-inflammatory agent and also inhibits the development of stomach ulcers.

Four new non-steroidal preparations are showing promise in clinical trials with arthritis patients. They are ibuprofin, tolmetin, fenoprogen, and naproxen. These drugs would probably be most useful for patients who cannot tolerate aspirin.

Heritable Diseases. Marfan syndrome, an inherited autosomal dominant trait, results in diffuse or generalized disorder of connective tissues. When patients die from this disease, it is usually due to an abnormality of the connective tissue of the large vessels of the heart. Collagen produced by fibroblasts cultured from individuals with this disease is more soluble than in normals.

Bone from patients with osteogenesis imperfecta has been studied for collagen structure. Data indicate that the bone collagen is overhydroxylated at its lysyl residues but not the prolyl residues.

Rheumatoid Diseases. In scleroderma there is increased collagen synthesis by fibroblasts. This increased synthesis is selective as overall protein synthesis is unchanged in normal and sclerodermic cultures. Even though there is selective activation of collagen synthesis, further studies must be undertaken to determine the mechanism of activation or regulation.

During the active stages of systemic lupus erythematosus, circulating antibodies to nuclear components of DNA are present in the blood. A grantee has recently developed a rapid, efficient method for measuring these antibodies in SLE patients. This test can be useful in predicting complications such as kidney disease days or weeks before clinical signs are manifest and it can lead to improved diagnosis and management of SLE patients.

In studying the pathogenesis of drug-induced autoimmune disease, hydralazine was shown to form physical complexes with deoxyribonucleoprotein - an action thought to be a mechanism for protecting deoxyribonucleoprotein from digestion with proteolytic enzymes in blood. This phenomenon perhaps may be a means whereby drugs may produce a lupus-like syndrome by the formation of antinuclear antibodies.

NZB-NZW hybrid mice are providing a good working model for systemic lupus erythematosus. A grantee using these mice has shown that the genetic defect in SLE is not a single one. A minimum of two factors - one genetic and one viral or both genetic - are thought to cause this disease.

Another scientist in studying viral antibodies proposes the hypothesis that SLE is a viral disease and those susceptible to it have a genetic defect which leads to the disease.

New findings linking ankylosing spondylitis with histocompatibility antigen W27 make it appear more likely that this form of arthritis is inherited and also that this condition is associated with other diseases. This antigen, although not directly involved in the disease process, is an excellent genetic marker and provides an explanation of many of the intricate patterns of the disease.

The goal of the arthritis program is to better understand the action of collagenases including mechanisms for controlling their biosynthesis and its structure of collagen in health and disease. Clarifying these mechanisms ought to increase our understanding of rheumatoid arthritis and osteoarthritis. The availability of a clearly defined training program with long-term stability would do much to guarantee a continued flow of scientists toward research careers in arthritis.

ORTHOPEDICS PROGRAM AREA

The area of research covered by the Orthopedics Program encompasses both basic and chemical studies of bones in normal and diseased states. It includes artificial implants with the related bioengineering, fractures and the healing process, transplantation and preservation of skeletal tissue, bone formation and repair, biochemistry of cartilage, as well as musculoskeletal dynamics and the physiology of normal and abnormal gait.

The orthopedics program currently has active 93 research grants and 2 program projects, 14 training grants, 5 career development awards, and 7 fellowships. The number of research grants supported by the orthopedics program has increased, especially in areas of artificial joints, bioengineering, and electric phenomena. Training support on the other hand has decreased, due to the phase-out of training programs starting two years ago. New applications for training grants are currently being reviewed.

Artificial Joints. Research and development of artificial joints has been progressing rapidly. The hip is the most frequent and successfully implanted joint to date. Not far behind, however, are the prostheses for knees, elbows and shoulders. Recently ankle joints and fingers have been successfully implanted. Materials for these artificial joints must be relatively non-toxic, non-antigenic, and display minimal wear and corrosion so that after a period of time few problems occur. Materials used include ceramics and plastics reinforced with graphite, steel, and various alloys. A recently developed high molecular weight polyethylene graphite material under vigorous testing appears the most promising at this time. The development of acrylic resins which cement the prosthesis to bone has enabled these advances to take place. These resins, however, display some undesirable effects and modified formulas are being tested which include development of pores within the cement to allow tissue ingrowth and thus hold the implant more firmly in place.

Scoliosis. This medical condition is being approached from various angles, including biplane radiography of the spine, testing of a telemetered Harrington Rod, oxygen consumption studies and pulmonary function tests, effects of exercise, and spinal monitoring during scoliosis surgery. Recently chickens with scoliosis have been bred. These animals are proving to be an excellent animal model of human scoliosis. One grantee in analyzing vertebral discs from scoliosis patients has demonstrated that acid phosphate levels were significantly increased and the glycosaminoglycan content significantly decreased throughout the area curvative of scoliotics.

A Scoliosis Workshop which was partially supported by NIAMDD was held in Chicago, Illinois, November 14-16, 1974. The purpose of this conference was four-fold: (1) to identify areas for research investigation to better understand, prevent, and treat scoliosis, (2) to determine the magnitude of the problem and establish better therapy, (3) to define the role of the Scoliosis Research Society in education and investigation of spinal problems, and (4) to develop mechanism of funding programs of research and education.

Osteoporosis. A five-year study is being conducted to evaluate the use of calcium, vitamin D, hormones (estrogens or androgens), diphosphonates and sodium fluoride alone and in various combinations in preventing and treating osteoporosis. Preliminary data are very promising. Hormones plus calcium supplementation appear to be the only treatment preventing osteoporosis. However, this treatment must be instigated prior to the onset of the disease. The combination which reverses resorption of bone once osteoporosis has started is that using fluoride and calcium. Preliminary results for this treatment indicate dramatic improvement.

Fractures and Healing - Electrical Current. Enhancement of bone healing through application of electric current is being carried on in numerous laboratories. Recently a grantee using a non-invasive electromagnetic treatment successfully promoted bone regeneration in five children with congenital-pseudoarthrosis, a disease extremely resistant to bone healing.

Studies with a proline analog, cis-hydroxyproline demonstrate that it can inhibit pertendonous adhesions (scars).

Cartilage. New micropuncture techniques have been developed by a grantee which permit the extraction of extracellular cartilage fluid from calcifying and non-calcifying zones of cartilage of experimental animals. Microanalytical methodologies for compositional analysis of this cartilage fluid have then been developed. These methods are powerful tools for studying proteins, proteoglycans and enzymes in interstitial cartilage fluids which open up a new world for exploration heretofore unavailable to researchers.

Bone Metabolism. The work on vitamin D metabolism and function by two grantees has uncovered new areas of investigation and new vistas for discovery. These findings have shown that vitamin D has several active intermediate forms. One of these 1,25 (OH)2 D3 was recently found to have two hormonal effects, one on calcium transport and a separate action on phosphate metabolism. These results shed new light in this area of research regarding mobilization of calcium and bone calcification. The newly discovered intermediate compounds are currently being tested for possible clinical use in conditions of hypocalcemia and hypophosphatemia and diseases such as osteomalacia and vitamin D resistant rickets.

Another grantee has reported that large doses of vitamin D can prevent and remedy bone disease caused by treatment of patients with corticosteroids.

The goal of the orthopedics program area is to understand the chemistry and physiology of bone formation and resorption and to continue to devise and perfect artificial implants for defective bones and joints. The availability of a clearly defined training program with long-term stability would do much to guarantee a continued flow of scientists toward research careers in orthopedics.

DERMATOLOGY PROGRAM AREA

In Fiscal Year 1975 a significant event occurred which directly concerns the Dermatology program area and one of its priority items. Training grant supported scientists including previously supported trainees reported clearing of psoriasis skin lesions in a significant number of patients who were given an oral dose of a photo-active drug, methoxsalen, and were subsequently exposed to long wave ultraviolet light. Although this type of interaction of light and drug, "photochemotherapy," is not a permanent cure for psoriasis, it appears to clear the skin lesions effectively and its repeated use seems to be well tolerated by the psoriatic patients who have been treated thus far. This method of management of psoriasis, however, has been tried for only a short time and it will now be subjected to intensive controlled investigation for long-term effectiveness versus late toxicity. This promising treatment does not preclude continuation of further basic or clinical research in psoriasis, a disease that is both physically and socially crippling to several million Americans. It is anticipated, therefore, that a contract to establish a psoriasis chemotherapy screening program will be awarded in June 1975 to one of ten competing university centers. The apparent defect in cyclic AMP enzyme regulation reported previously to be disturbed in psoriasis is still a valid and primary consideration, and will be actively pursued within the context of a new program project grant awarded to the University of Michigan School of Medicine.

Broad ranging studies in psoriasis will combine the efforts of investigators of several departments and will include research crossing the fields of dermatology, biochemistry, pharmacology, pharmacy, and anatomy. The investigations will continue to place emphasis on the role of cyclic nucleotides in the control of epidermal proliferation, but will also initiate studies of other facets of the disease. This group at the University of Michigan was the first to describe altered hormonal controls in the skin of patients suffering from psoriasis. Within the same program project a collaborative project will be carried out at the University of Utah. This will be directed toward the detection of cell-mediated immunity in psoriasis. An attempt will be made to formulate an in vitro model of psoriasis utilizing transplantation of normal and psoriatic skin to germ-free congenitally athymic mice.

As reported in the FY '74 annual report, the Dermatology Program Director and the Chief of the Allergy and Immunology Branch of NIAID conducted discussions regarding the possibility of a jointly sponsored Immunodermatology Workshop. Plans for this workshop to be held in October 1975 have been finalized. Approximately forty scientists, many of them young, representing the disciplines of allergy, immunology, and dermatology, will discuss IgE-related cutaneous problems including atopic dermatitis and urticaria, immune complex diseases, and complement in cutaneous diseases including vasculitis and bullous disorders, cellular mechanisms and delayed hypersensitivity including cutaneous infections and immuno-deficient states. An attempt will be made to have the proceedings of this unique workshop published within six months following the meeting.

Significant advances have been made by grantees in the following areas: sebaceous gland physiology: much of the work concerning the effect on the sebaceous gland from the exogenous administration of hormones has been completed by our grantees. Among the more important findings have been the observations that (a) estradiol-17β applied locally to the skin does not reduce sebaceous secretion as was noted to occur previously with ethinyl estradiol; (b) sebaceous secretion in women can be stimulated by the administration of the anabolic agent, 17α-ethyl-19-nortestosterone, which is a weak androgen. Thus, sebaceous secretion in women is not maximally stimulated by endogenous androgen as it almost always is in men, and further, that the reason for developing acne after the use of this anabolic agent is undoubtedly its effect on sebaceous gland secretion; (c) topical administration of 10% testosterone propionate to the skin after estrogen administration does not reverse sebum production to the pre-estrogen treatment level, unlike the stimulating effect induced with systemically administered testosterone, suggesting that some local inhibition by estrogen is possible. These same grantees report that since their previous report of one year ago, no anti-androgens have become available for study.

Biochemistry of altered dermal connective tissue: grantees in Philadelphia have reported previously that the induration of the skin in scleroderma was primarily due to the replacement of the subcutaneous tissue by newly synthesized connective tissue. In order to confirm the above findings investigations were further expanded and preliminary information shows that replacement of subcutaneous tissue by connective tissue is a general phenomenon in all forms of scleroderma. It is now clear that in systemic sclerodermatous skin there is a marked increase in the absolute content of collagen due to the replacement of the subcutaneous tissue by newly synthesized connective tissue. This increase of collagen is accompanied by an increase in the fibroblastic population of the subcutaneous tissue. In localized scleroderma the deposition of collagen is preceded by an inflammatory reaction in the subcutaneous tissue consisting of numerous plasma cells and lymphoid follicle formation. This finding suggests an immunologic response.

Action of ultraviolet radiation on cells: ultraviolet radiation is a significant ecological factor. Grantees in Texas are examining the mechanisms by which ultraviolet radiation inhibits the growth of bacteria, since this light can kill and mutate bacteria; its effects on man are even more obvious. In man ultraviolet induces sunburn, sun tan, skin cancer, and various allergic and photodynamic diseases and it produces Vitamin D from sterols in the skin. It has not been determined what site or mechanism within the cell is the target for UV radiation that induces growth delay. However, grantees are able to report that their studies on macromolecular synthesis after UV radiation indicate that RNA synthesis is more sensitive than DNA or protein synthesis, and this suggests that UV is probably hitting systems in the cell that are concerned with RNA synthesis or translation. It also appears likely at this time that UV radiation is acting at the ribosomal level, where it is interfering with mechanisms that control the rate of RNA synthesis.

DIABETES PROGRAM

The Diabetes Program during FY 75 supported approximately 160 research grants, five Diabetes-Endocrinology Center Grants, as well as a variety of grants in support of training. The total budget was $12,977 million.

One of the major areas of grant support by the diabetes program is centered around structure-function studies of insulin and related compounds, as well as of related peptides and enzymes, and studies of the mode of action of insulin and glucagon including the mechanisms of release, secretion, and biosynthesis. An area of intensified interest during this year has been studies on the action of somatostatin, a peptide hormone of the hypothalamus. The action of somatostatin to inhibit secretion of both insulin and glucagon (as well also of growth hormone) has provided a new tool for studying in greater depth the roles of insulin and glucagon in the pathogenesis of diabetes. Onset of action of somatostatin is extremely rapid and its biological half-life is extremely short, but "long-acting" preparations are becoming available. Whether or not somatostatin becomes a part of the therapeutic regime, it will have an important role in further elucidating metabolic interrelationships relative to the pathophysiology of diabetes.

Another important area of research support centers around studies of carbohydrate metabolism and gluconeogenesis, lipid metabolism, nutritional relationships, and general metabolism related to diabetes. Important studies in capillary permeability and basement membrane thickening are also supported.

There is also a significant amount of support for clinical studies dealing primarily with the pathophysiology and biochemistry of diabetes. The only large clinical trial, the University Group Diabetes Project (UGDP), now in its 15th year, was concerned with long-term use of oral antidiabetic agents in patients with maturity-onset diabetes. In 1970 they reported significantly increased cardiovascular mortality in patients on the oral agents. Controversy resulting from this report led to an NIAMDD-supported independent reassessment of the results by a committee of the Biometric Society. This report, published this spring in the JAMA, supports generally the UGDP report, but calls for more research to adequately evaluate those drugs.

An important area of research which has gained considerable emphasis recently is the prospect of transplantation of islet cells of the pancreas as a mode of therapy. In addition to work on actual transplantation procedures in animals, considerable support is also awarded for basic physiologic studies of the islets and β-cells, including work on cell cultures. While the concept of islet cell transplantation (or whole organ transplantation) appears to be very exciting and may eventually be fruitful, the NIAMDD in conjunction with the American Diabetes Association has been compelled to sound a warning. Such transplantation, of whole organ or islet cells, is presently a highly experimental procedure and further extensive animal experimentation must be conducted to assure safety. Such research in human beings is regarded as premature because of the serious problems of tissue incompatibility. Transplantation, along with development of devices which may lead to another mode of treatment, the "artificial pancreas," received considerable attention during this year in the public press. Considerable additional development of such devices is needed before they might be ready to be tested as a feasible therapeutic approach. At present, the Diabetes Program does not support any research directed toward the development of an "artificial pancreas."

Support continues to be awarded for development and study of animal models. Epidemiological research is being carried out at the Field Station in Arizona (described elsewhere in this report). Evidence continues to emerge to indicate that diabetes may not be one disease, or, at least, may result from a variety of causal factors, including autoimmunity and viral etiology, both of which may be separate from or acting concomitantly with genetic predispostion. Some studies are currently being supported in this area.

During this year one conference was partially supported (by direct funds) on "Neural Pancreatic Interactions." Another important activity relating to the Diabetes Program is the publication by the NIAMDD of the Diabetes Literature Index (covered elsewhere in this report).

In November 1974 the first awards were made for Diabetes-Endocrinology Centers. These Centers now number five, and are located at Vanderbilt University, University of Virginia, University of Chicago, University of Washington (Seattle), and Washington University (St. Louis). The Centers are designed to expand ongoing fundamental and clinical research efforts in diabetes, and to expedite the flow of information from basic scientific studies into clinical applications. The Centers are established only where a substantial, productive program in diabetes already exists. During this fiscal year diabetes gained renewed national attention with the passage of the "National Diabetes Mellitus Research and Education Act" PL 93-354, in July 1974. This Act provides for a National Commission on Diabetes consisting of six scientists involved in diabetes research, four lay members, two of who must have diabetes or be the parent of a child with diabetes, and seven Institute Directors (NIAMDD, NEI, NHLI, NIDR, NINCDS, NICHD, and NIGMS). The Commission is charged with producing within nine months a long-range plan to combat diabetes with specific recommendations for the utilization and organizatin of national resources for that purpose. The Commission began its deliberations on March 10, 1975, and, thus, their report will be due December 10, 1975. At this time the Commission has met four times, and is aggressively pursuing its tasks.

The Act also mandated the formation of a Diabetes Mellitus Coordinating Committee (DMCC), which includes representatives from the seven Institutes named above, and representatives from other Federal agencies with diabetes-related activities. This Committee is charged with reporting their progress in coordination of diabetes-related activities to the Director, NIH, at the end of each fiscal year. This committee has been formed, thus far has met twice, and is working on the first report. While the Commission is in existence, the DMCC also serves as an important resource for the Commission. The Diabetes Program Director, NIAMDD-EP acts as Executive Secretary for both the Commission and the DMCC.

Endocrinology Program Area

The Endocrinology Program supports basic and clinical research on the structure and function of the endocrine glands, the hormones secreted by these glands, on other factors with hormone-like activity, the mechanism of action of the hormones and the effects of other factors involved in the action of hormones. For fiscal year 1974, the program supported about 257 research grants with an encumbered amount of $15.2 million. We also supported various fellowship programs including Research Career Development Awards and Research Career Awards. The total of these awards was 66, for an encumbered amount of $1.2 million. Finally, the Endocrinology Program supported 38 training grants, with a total encumbered amount of $2.4 million. A total of 24 of the new training grants (T32) are being reviewed by the June 1975 Advisory Council.

In last year's report, it was indicated that an evaluation of the National Pituitary Agency was being conducted by contract with the National Academy of Science. The evaluation has been completed and a report submitted. The recommendations are presently being reviewed by senior NIAMDD staff for possible implementation.

Recent studies involving the endocrine glands are exemplified in the following categorical classification.

Pituitary

Growth hormone from the pituitary gland appears to exert its effects through a component of plasma which has been named somatomedin. Recent studies have shown that somatomedin stimulates growth in a variety of tissues other than in the skeleton. Somatomedin appears to be a powerful mitogenic agent, which might shed light on the etiology of cancer. Recent studies have also shown that there is a new class of peptides which includes epidermal growth factor, nerve-growth factor, erythropoietin, multiplication stimulating activity, fibroblast growth and possibly other growth activities. Researchers have been able to show that somatomedin has its own unique binding site in placental and liver cells. A radioimmunoassay using placental membrane preparations and ^{131}I somatomedin C, has been developed. This assay is capable of measuring accurately the somatomedin levels in unextracted blood plasma.

Work on growth hormone has also shown that when residues 135-146 were removed from the human growth hormone, the growth-promoting activity of the hormone very nearly tripled. It has also been found that a potent diabetogenic factor is an enzymatically modified growth hormone. This will permit re-examination of the relationship of growth hormone to diabetes.

Studies are going on concerning the primary structure of human follicle stimulating hormone. The alpha and beta subunits of human FSH have been isolated in a highly purified form. The entire primary structure of the alpha and beta subunits has now been determined. The alpha subunit is identical to the alpha subunit of luteinizing hormone and thyroid stimulating hormone. The beta units have many similarities, but are not identical. Studies are being undertaken to elucidate the nature of the carbohydrate moieties of human FSH and LH, as well as the reactive groups. Chemical modifications of these molecules are planned to ascertain which structural characteristics determine the specific nature of the biological and physiological activities of these two hormones.

Systematic studies have been made of the biosynthesis and secretion of ACTH in cell culture. The researchers feel that once the pathway of ACTH production is known, it should be possible to pinpoint the site of action of substances that regulate ACTH production. Three molecular forms of ACTH have been found in a mouse pituitary cell line and in extracts of bovine, mouse and rat pituitary glands. It appears that the major biological activity resides in the smaller form of the hormone, a molecule of about 39 amino acids.

Other studies have shown that the growth response of mammary cancers in rats to prolactin was related to prolactin receptor activity. This might prove to be a valuable observation in that human breast cancer biopsies could be tested for prolactin receptors to determine whether or not it would be advisable to treat patients of this type with prolactin-inhibiting factor or some other prolactin-inhibiting drug. In addition, other animal studies have shown that breast cancer can be prolactin-dependent. There have also been reports of an increased incidence of breast carcinoma in patients with thyroid diseases. Therefore, studies have been instituted in hyper- and hypothyroid patients to see, if the prolactin levels are changed. It was found that the basal levels of prolactin were normal, but in hypothyroid patients, there was a marked increase of prolactin response to thyrotropin-releasing hormone, and in the hyperthyroid patients, there was a blunted response to TRH.

Thyroid

The mechanisms of action of the thyroid hormone at the cellular level is still largely unknown. Several recent studies, however, have given us more insight into this activity. For example, both hyper- and hypothyroidism will influence the response of hormones which act through cyclic AMP-mediated systems in the cell. The responses to parathormone, glucagon and epinephrine have been shown to be exaggerated in hyperthyroidism, and blunted in hypothyroidism. Another investigator has shown that there is a T_3-binding site in lymphocytes of normal humans. He was also able to show that in one patient with well-documented peripheral resistance to the action of thyroid hormone, the lymphocytes did not have the usual high-affinity binding sites for thyroid hormone. This seems to indicate that the nuclear thyroid hormone binding protein - T_3 complex may definitely play an important role in the mediation of thyroid hormone action.

It has been known for many years that the thyroid hormone molecule is found in two forms: so-called tri-iodo-thyronine (T_3) and tetra-iodo-thyronine (T_4). As the names imply, these substances contain three and four iodine molecules, respectively. It is not known exactly what the physiological relationship of these two forms of the hormone is. Recent studies on the effect of starvation on peripheral thyroid hormone economy suggest that starvation is associated with a prompt and pronounced decrease in the conversion of T_4 to T_3. The researchers feel that this could be a shift to production of "reverse T_3." Another investigator has found a decrease in circulating T_3 in a number of patients hospitalized for various reasons. He has noted this shift in 70% of the patients examined and feels that this could be due to a decrease in the conversion of T_4 to T_3.

Another investigator reports the development of methods of measurement of tissue concentrations of both T_3 and T_4 using alcohol extraction and immunoassays, which has permitted him to show that T_3 in the body is predominantly intra-cellular and that T_4 is predominantly found in the blood. He notes that the respective pools in blood and in cells are in equilibrium. As an important consequence of this, he has found that in lung there is an unusually high T_4 and T_3 concentration and that this hormonal content develops on the day of birth, when it coincides with the appearance of pulmonary surfactant. This group feels that maturation of surfactant may be dependent upon the ability of the lung to concentrate T_4 from the blood. This may be a very important observation in view of the fact that respiratory distress in infants has been shown recently to be related to low plasma T_4.

A triiodothyronine test has been developed which can clearly separate patients with primary hypothyroidism from euthyroid patients. Other studies have now been completed in which the amino acid sequence of human thyroxin binding pre-albumin has been determined. Finally, it has been shown that thyroid hormone exhibits a positive feed-back on the hypothalamus while it inhibits the pituitary. This has been shown by the measurement of TRH excretion in urine.

Adrenals

Adrenal steroids, which control mineral and water metabolism, are involved in almost every major regulatory process in the body and appear to be central to the responses to trauma, inflammation and stress. Understanding the factors involved in the production of these steroid hormones is clearly of major medical importance. The P450 catalyzed oxidation of cholesterol is thought to be the rate-determining step in corticosteroid synthesis and the principal site of ACTH control. Studies are being supported in which attempts are being made to solubilize and purify the adrenal P450. It has also been shown that P450 can convert cholesterol sulfate to pregnenolone sulfate. This is an important reaction because fetal and malignant adrenal cortex secretes steroid sulfates which may be formed from cholesterol sulfates. Studies of this sort, in addition to giving us information concerning the methods of action of the various hormones, may also provide some insight into the malignant process. Other investigators have found that an inhibitor of adenyl cyclase blocks steroidogenic action of angiotensin in the rat adrenal. The reaction appears to occur through inhibition of the 3-beta-hydroxy steroid hydrogenase and thus curtails cortisol and aldosterone hypersecretion in man.

Parathyroid Hormone and Calcitonin

These are the hormones responsible for the balance of calcium in the body. They are of particular interest clinically because of the role they play in osteoporosis and in chronic renal failure. The amino acid sequence of parathyroid hormone (PTH) has been reported previously and been confirmed. Studies are now under way on both PTH and calcitonin receptors in various cells of the body. There is evidence that shows that bovine parathyroid slices secrete another protein which responds in parallel with the PTH to changes in the concentration of calcium in the medium. What this protein is, is unknown at this moment. However, it may be a neurophysin-like protein, it could be an enzyme that converts pro-parathyroid hormone to PTH or it could be a high-molecular weight form of PTH. Other possibilities are that it could be a calcium-binding protein or a structural protein related to the secretory granule. Further studies are expected to elucidate what this parallel type protein is. There seem to be various forms of the PTH. Several of them have been detected in the circulation. Some evidence suggests that there is a peripheral cleavage of the intact PTH once it is secreted. One group of investigators has found the site of cleavage, and found that amino acids 1-33 appear to be active parts of the hormone molecule. In order to study the various forms of the PTH, attempts are being made to develop immunoassays to these various forms in order to follow them in their metabolic pathways.

Gonadal Hormones

Vasectomy is one of the methods used for population control. However, other than causing sterility in the male, the effects of this operation over a long term are essentially unknown. Consequently, a study was done on the long-term effects of vasectomy in man. Plasma testosterone, FSH and LH and the percent of binding of testosterone to plasma protein were studied in 16 normal fertile males and in 81 males who had been vasectomized from 1 to 5 years. There was no significant difference in the hormone levels of the two groups.

By using a fibroblast binding assay for 3H dehydrotestosterone-binding in genetic males with a female phenotype, it was shown that there was no detectable specific 3H-DHT binding. This is the first report of a defective or absent intracellular steroid hormone receptor tissue in man.

Researchers have confirmed that it is possible to inhibit secretion of estrogen and progesterone from the corpus luteum and possibly from the placenta in early pregnancy by specific inhibitions of cytochrome P450. This raises the possibility that inhibition of this enzyme could be useful as a contragestational agent or as an inhibiting factor in steroid-secreting malignancies.

Hypothalamus

It is now well established that the nervous system interacts with the endocrine system. One of the major sites of interaction is in the hypothalamus of the brain stem. There are numerous studies being supported which relate to the identification and synthesis of various of the releasing factors from the hypothalamus. In addition, physiological studies are being supported with the hope that they will elucidate the mechanism of action of the releasing factors.

One researcher has demonstrated a potent corticotropin releasing factor in the peripheral blood of rats. He has named this factor tissue CRF. It can be elicited in rats with hypothalamic lesions and has a high-potency and prolonged action in recipient animals. They believe it to be different from the CRF of median eminence origin.

Molecular Mechanisms

Much of the important research in endocrinology is concerned with basic studies of the molecular mechanisms involved in the action and interaction of various hormones, receptors and second messengers such as cAMP. There is increasing evidence that cAMP is involved not only in the physiologic function of hormonal tissue but may be a controlling function of cell-growth regulation and may mediate abnormal cellular function in certain pathologic states. There is also increasing evidence that cGMP opposes cAMP in affecting several biological processes including growth. Investigators are attempting to identify cGMP receptor protein and separate it from cAMP receptor protein. One group is utilizing cell culture techniques to clarify the molecular mechanisms involved in the action of thyroid hormones on mammalian cells and will relate these findings to the general question of cellular control mechanisms in higher organisms and man. Their studies on interaction of thyroid hormone with cellular receptors have shown that T_3 appears to control prolactin as well as growth hormone secretion in their system. Another group is studying cellular calcium homeostasis which will hopefully contribute to a better understanding of the systemic calcium homeostasis and its regulation of PTH, CT, vitamin D and phosphate. Based on available data, a computer model has been developed which suggests that calcitonin regulates the cell calcium metabolism and inhibits the transcellular calcium by stimulating the rate of calcium uptake by mitochondria.

These few examples just scratch the surface of the basic research being supported in the area of endocrinology, the importance of which cannot be emphasized enough.

Medical scientists have been challenged for some time to determine why the incidence of cardiovascular disease increases so much in Chinese and Japanese when they adopt American diets. Scientists in the Metabolism Program suspect the culprit is methionine and that susceptibility to its toxicity is genetically determined. They contemplate a new breed of counselor trained in enzymology, nutrition, genetics, and screening technology who can identify the susceptible individuals and provide for each a diet adequate for normal growth and metabolic challenge (such as pregnancy) for each stage of life. Hormones direct intermediary metabolism to satisfy bodily needs but the consequences of many inborn errors of metabolism can be avoided by strict control of the diet.

A key role for methionine in metabolic regulation has been recognized for some time since it is an essential amino acid required not only for protein synthesis but for various alkylation reactions. Methylation of proteins and nucleic acids is a form of post translational control since their susceptibility to enzymatic degradation can be profoundly altered by the methylation. Aminopropylation converts putrescine (derived from ornithine by decarboxylation) to spermidine and then spermine. The cell content of spermine correlates closely to its DNA content while that of spermidine is related to RNA content. Spermine reinforces the cross linking of the double helix and thus represses the genome while spermidine reinforces the complementary regions of the RNA loops and facilitates the formation of single stranded DNA which is thus derepressed. It works synergistically with magnesium in RNA polymerization and protein synthesis. Nucleic acid synthesis can proceed temporarily in the absence of polyamine synthesis but the rate of protein synthesis and cell growth falls off rapidly.

Normal individuals maintain almost constant levels of methionine in their body fluids whether in feast or famine and its uptake by tissues peripheral to the liver is subject to strict control by a variety of hormones. This fine balance depends on its rapid conversion in the liver to S-adenosylmethione which comes into equilibrium with S-adenosylhomocysteine, homocysteine, adenosine, and a variety of addition compounds of homocysteine. Excess methionine is detoxified by conversion of the equilibrium amount of homocysteine to cystathionine (an irreversible reaction) which is further converted to cysteine and ketobutyric acid. Deficiencies in a number of enzyme activities can lead to elevations in homocysteine levels. Severe homocystinuria is associated with early severe atherosclerosis and early death. What is now being recognized is that the macro- and microvascular pathology of diabetes and atherosclerosis is comparable to that of homocystinuria and that similar pathology is found in rats fed homocysteine. Such rats continue to grow after hypophysectomy (with thyroid replacement) and even produce somatomedin in the absence of growth hormone.

Trace amounts of homocysteine have been observed in the plasma of cardiovascular patients but this may be only the tip of the iceberg since it has now been recognized that homocysteine can form a host of derivatives, some of which are fat soluble and transported in the lipoproteins of the blood. Transport in this manner bypasses the hormonal regulation of methionine availability and permits undesirable tissue proliferation. Current analytical procedures are inadequate for extensive studies but the use of stable isotopes which can be measured by mass spectroscopy should open the field to more extensive investigation. It will be most interesting to learn if the Pima Indians with their high incidence of diabetes show elevated homocysteine levels.

The second exciting development concerns the role of polyamine oxidation products in metabolic regulation. Until recently the polyamines were considered dead end metabolites whose only fate was excretion from the body. Now the evidence is incontrovertible that the primary amine of the aminopropyl residue can be converted to the aldehyde whether by transamination or oxidation. These β-aminoaldehydes decompose readily under physiological conditions of temperature and pH to form acrolein (vinylformaldehyde) and the next lower polyamine. Spermine is thus converted to spermidine and then to putrescine and each molecule of acrolein is ultimately derived from one molecule of methionine or homocysteine. It is now apparent that this shuttling between putrescine and spermine is significant and probably plays a fundamental role in the induction of protein synthesis (by converting the gene repressor spermine to the gene derepressor spermidine) and its post translational control.

The post translational control of protein synthesis appears to reside in the capacity of acrolein and the aminoaldehydes to act as analogs (and thus either competitors or synergists) of important cell constituents. Acrolein is vinylformaldehyde and thus the analog of formaldehyde which plays such a fundamental role in one carbon metabolism and the folate-vitamin B12-S-adenosylmethionine methylation complex. This complex in its active state of aggregation contains four molecules of pyridoxalphosphate as structural components. It is noteworthy that many of the glucocorticoid induced enzymatic activities utilize pyridoxalphosphate in either a structural or functional capacity. The aminoaldehydes also appear to be transition state analogs of glucose for the glycosidase enzymes. The high energy transition state is thought to involve a ring oxonium ion which is simulated by the charged amino group of the aminoaldehyde. Exploration of the interactions of the aminoaldehydes is complicated by their extreme reactivity and instability. Almost any change in pH, temperature, carbon dioxide tension, concentration, or metal ion activity can result in extensive redistribution amongst a variety of species in thermodynamic equilibrium with one another. Thus, investigation of the role of the polyamines in chemoreceptors is particularly inviting.

Most cell components are in thermodynamic equilibria with each other as a result of both enzymatic and non-enzymatic reactions. This means that keto acids and hydroxy acids are in equilibrium with the corresponding amino acids as determined by the activity of ammonia and the redox potential of the pyridine nucleotide couple. Divalent cations come to equilibrium with a variety of chelate agents such as ATP or citrate and are thus limited in their availability for interaction with metal dependent enzymes.

Because of their great reactivity aldehydes are thought to come to equilibrium with amines to form Schiff bases (aldimines) without intervention of enzymes. Most amines have pKa's well above the physiological range and are not available for Schiff bases formation which requires the free amine. The presence of a strong basic charge as of the guanidino group of arginine or the sulfonium charge of S-adenosylmethionine can weaken the basicity of an adjacent amino group so it is now available to react with aldehydes. A variety of enzymes have weak amino groups as a result of such charge interaction. These enzymes are readily inhibitable by pyridoxalphosphate and comparable inhibition by aminoaldehydes needs investigation.

The capacity of aminoaldehydes to act as analogs of the divalent metals may have equally important consequences. Divalent metals are intimately involved in the activation of the various enzymes of cyclic nucleotide metabolism such as the nucleotide cyclases, phosphodiesterases, protein kinases, and the protein phosphatases. Each enzymatic reaction also influences the metal activities through changes in the concentrations of such chelate agents as ATP, ADP, pyrophosphate, inorganic phosphate, and the cyclic nucleotides themselves. The metals are also involved in the aggregation-deaggregation reactions of enzymes which are known to control their activities. Metabolic regulation of this type is observed in primitive forms of life with little or no involvement of hormones. The evolutionary process led to survival of those enzymes which showed an appropriate response to the changing nutritional requirements and supply of the organism. It seems apparent that hormones took over control of these basic interactions by providing better targeted messengers for the metabolic messages.

The cystathionine pathway for degradation of methionine requires thyroxine. A number of observations suggest that the polyamine aldehydes act as feed-back inhibitors to their own synthesis (S-adenosylmethionine reacts with aldehydes but is also involved in polyamine synthesis) and in the smooth adjustment of enzyme activities from anabolic to catabolic and return. In the absence of such regulation the enzymes work counter to each other (futile cycles) as in the phosphorylation and dephosphorylation of the same substrate with consequent waste of ATP energy as heat. By opening the cystathionine pathway thyroxine appears to decrease the intensity of the polyamine damping of the enzyme complexes with a consequent increase in the basal metabolic rate. Many interrelationships between the polyamines and such hormones as prolactin, insulin, growth hormone, thyroxine, and the steroids have been observed in the mammary gland explant, the adipocyte, and the tissue culture fibroblast. It is apparent that the polyamines and their degradation products have been the neglected parameters of metabolic regulation.

The nature of the basic defect in Cystic Fibrosis is still obscure but the most immediate effect seems to be the escape of polyamines from various exocrine cells along with their secretions. Extracellularly they can be converted to β-aminoaldehydes which can compete with calcium for various binding sites. Any primary secretion is essentially isotonic with blood but the final secretion has been modified by the active transport of the various solutes. Resorption of solute from lung secretions is particularly important since the free water is required to humidify the inspired air which for most animals (non-sweating) is the basic means of temperature regulation. This resorption process depends on passive entry of sodium into the pump cell and its active extrusion into the lateral spaces between the epithelial cells. This space is normally insulated from the mucosal surface by a cement dependent on calcium for its integrity. Apparently the aminoaldehydes displace the calcium, increasing the permeability of the membrane. Accordingly water follows the pumped sodium through this short circuit resulting in the highly dehydrated mucus of high calcium content characteristic of CF lung secretions.

Research on CF has been characterized by almost invariable failure of one laboratory to duplicate the results of another at first try. It has proven necessary to standardize on site of biopsy, age, sex, composition of tissue culture medium, method for dispersion of cells, analytical methods, etc. Particularly important may be the choice of animal species from which tissue culture serum is prepared and its concentration since the various species differ in their content of polyamine oxidases and the carriers for the aldehyde oxidation products. These factors change markedly after birth and in ruminants on development of the rumen. A second factor is whether or not a primary amine buffer such as Tris or Tham is used. Tris has a pKa in the vicinity of 8 and is usually present in high concentration so it has considerable capacity to trap aldehydes and block their effects. The effects of Tris are best known for the enzymes with weak epsilon amino groups essential to their function so extrapolation of such a mechanism to coordination of the metabolism of the whole cell has some justification.

The perfection of the fiberoptic bronchoscope should markedly facilitate the study of the lung complications of CF. The capacity to introduce or withdraw fluids permits the introduction of possible etiological agents for the lung pathology and the testing of possible therapeutic agents such as mucolytic agents. Substitution of such manipulation for bronchial lavage with its hazards and complexity is also a promising prospect.

The Metabolism Program has had $20,476,000 available for research grants during the year. It provides the major support for the very basic studies of metabolism which are necessary to understanding the nature of disease and its management. It should be apparent that these studies have been essential to unravelling the complexities of methionine metabolism and that much remains to be done in applying this knowledge to management of disease.

HEMATOLOGY PROGRAM AREA

A major thrust of this extramural grants program is into research in the fundamental areas of structure, function, metabolism, and properties of red and white blood cells, platelets and their components, of plasma proteins and hemoglobin, and of facets of immune system. There is a strong bearing of this basic research upon hematologic disorders including: hemoglobinopathies such as Cooleys or thalassemia, and sickle cell disease; hemolytic, aplastic, megaloblastic, and pernicious anemia, and the purpuras. Eighty percent of the hematology grants moneys are devoted primarily to basic aspects of research while twenty percent are more disease-related although there is a good deal of inter-face between these two aspects. In surveying the spread of these grants the inter-relationship of hematology to many other disciplines is impressive as is the diversity of its areas of endeavor. The funds expended on hematology represent almost ten percent of the NIAMDD research grant expenditure.

Erythrocytes, Erythropoietin, Hemoglobin and Iron

The availability of the red blood cell for study has facilitated studies of its formation, metabolism, survival and destruction, as well as of the components related to its function such as hemoglobin, iron, and erythropoietin. Almost sixty percent of the more fundamental hematology research funds are devoted to this area. Some of those studies bear upon transplantation, anemia of renal failure, renal and other membrane function, as well as upon general and erythrocyte metabolism. Metabolic activity and membrane ability of fetal and adult cells are being compared in one laboratory to delineate the mechanism by which glycolysis is eliminated from the red cell at birth. Several laboratories are investigating aspects of erythropoietin - production by kidney cells in tissue culture; mechanisms of formation and secretion; extra renal erythropoietin production in experimental animals and in patients with chronic renal disease. Red blood cell Ca, Na, K and membrane ATPase activity are being explored in several laboratories in which correlations are being made between red cell and renal cell transport as well as with manifestations of changes in diseases such as hereditary spherocytosis, sickle cell anemia, cystic fibrosis and end stage renal disease. Over a third of the funds in this area are devoted to hemoglobin studies: genetic control of hemoglobin structures, amino acid sequence in zeta chain of human hemoglobin, correlation of structures with altered chemical properties and biologic function, studies in Belgrade rats which have a hereditary thalassemia-like anemia, structural aspects of protein allosterism in human hemoglobin with study also of binding of drugs of potential value in treatment of sickle cell disease, the erythroferrin role in hemoglobin synthesis, etc. Iron, isoferritin, tissue acceptance and release of iron in vivo and in vitro and the related regulatory mechanisms are under study.

Anemias and Hemoglobinopathies - Sickle Cell Disease and Thalassemia

Over sixty percent of the more disease related grants' funds are devoted to study of Cooley's Anemia and of sickle cell disease. The NIAMDD is represented on an Inter Agency Cooleys Anemia Committee (which coordinates activities of NIH and other relevant government agencies) by the Associate Director for Kidney, Urologic and Blood Diseases who serves as Vice Chairman, and by the former Hematology Program Director who serves as Executive Secretary. Over a million dollars is devoted to grants relating to Cooley's Anemia, many of which elucidate mechanisms of hemoglobin synthesis, regulation, genetic control, of iron storage, metabolism, and mobilization, etc. Four contracts have been awarded for development and testing of iron chelating agents in the treatment of Cooley's Anemia. Studies of sickle cell disease include those of the sickling mechanism, chemistry of sickle cell anemia variants, of cyanate therapy, xray crystallographic structure of sickle hemoglobin, iron loading in sickle cell disease, etc., as well as the studies of hemoglobin which compare many facets of hemoglobin with Hb-S. Research grants about hemolytic, aplastic, megaloblastic and pernicious anemias make up over 25 percent of the funds for disease-directed grants. They include enzyme studies of 6-6-PD; of superoxide dismutase; studies of isohemagglutinins, of auto-immune hemolytic anemia, folic acid metabolism, folate-bindary protein; B-12 kinetics, etc.

Immunologic Hematology

Auto-antibodies, auto-immune disease, antigens, antibodies, complement, the reticulo-endothelial system and transplantation immunology comprise over a quarter of the expenditures for more fundamental studies in this sector. HL-A antigens in disease, immunosuppression, tissue alloantigens, antibody affinity in immuno response and tolerance, regulatory mechanisms in immunobiology and 1GA globulins comprise one grouping of grants. Six grants approach different aspects of complement: Protein C1, C2 esterase; cellular synthesis of complement components, structures and function of C5, etc. Nine grants are involved with evaluation of transplantation: tissue specificity, chemistry and development of transplantation antigens, graft-host interaction, homograft reactions, characterization, anti-vascular endothelium antibody, humoral antibodies, transplant rejection and C-3 conversion inhibition, etc.

Miscellaneous

There are another group of grants that include bone marrow studies, coagulation factors, plasma and serum proteins and white cells in their purview. Leucocyte studies include those of maturation and function, ultrastructure, genetics, regulation of differentiation and enzyme studies. The bone marrow grants run the gamut from electrolyte interaction and enzyme regulation to studies of injury and repair, of cell cycle of hematopoietic progenitor cells and of bone marrow kinetics.

OFFICE OF THE ASSOCIATE DIRECTOR

FOR

KIDNEY, UROLOGIC, AND BLOOD DISEASES

ASSOCIATE DIRECTOR'S REPORT

The Kidney, Urologic, and Blood Diseases Cluster includes two programs, of which one, hematology, has been without a program director since mid-January, 1975. The cluster has been involved in establishing informal relationships with other NIH groups: an epidemiology discussion group has been initiated; discussions with intramural investigators involved with nephrology and urology research, and with extramural program directors responsible for administration of renal and/or urologic grants/contracts.

The Associate Director is Co-chairman of the Advisory Committee on Epidemiology and Statistics of the National Kidney Foundation which is ferreting out available, or potentially available data, about kidney and urinary tract disease. As a result of this activity, several courses of action have been recommended which can assist the cluster in obtaining data which should prove helpful in determining program relevance of disease areas. Tapes have been obtained from the Army and Air Force, and those from the Navy will be obtained, which will be analyzed for the incidence and prevalence of kidney and urinary tract disease, and demographic characteristics of the denominator population of servicemen admitted to medical facilities for treatment of kidney and urinary tract disease. Exploration of the possibility of retrospectively analyzing data already collected by states treating patients for end stage renal disease, by prepaid health groups, etc., is being undertaken. Possible collaboration with other institutes of NIH is under consideration in order to minimize duplication of effort in collection of epidemiologic data. Some of the information to be obtained could be utilized by the Evaluation of Research Needs in Nephrology and Urology, noted below.

A contract has been awarded to Drs. Gottschalk and Lassiter of the University of North Carolina as principal investigators for a study, Evaluation of Research Needs in Nephrology and Urology. This contract has the following five aims:

1. Assess the state of the art in research in the respective and inter-related fields of nephrology and urology.

2. Delineate the needs for future basic, clinical, and applied research in nephrology and urology.

3. Evaluate the feasibility of carrying out the research in the areas considered significant by scientists active in the field; and the technical and methodologic advances necessary to implement the required work.

4. Consider priorities in approach to the different recommended areas based on: a) need for elucidation of basic mechanisms of disease in order to devise rational treatment; b) clinical consideration

of morbidity and mortality; c) epidemiologic data; and d) fiscal considerations.

5. Produce a detailed working paper resulting from the study to be distributed to interested and involved persons throughout both the scientific and lay community of the public and private sectors.

There are eleven specialty committees which are responsible for the following areas: Acute Renal Failure, Chronic Renal Failure, Circulatory and Hematologic Disorders, Glomerulonephritides, Immunopathology, Normal Kidney Structure and Function, Obstructive and Neuromuscular Disorders, Pediatric Nephrology and Urology, Sundry Conditions, Urinary Tract Infections, and Urolithiasis. The chairmen of the committees, coordinating committee, principal investigators, and project officer met in mid-May 1975 to discuss progress in the study, as well as adherence of the committees' work to aims, schedules, etc. Most of the committees have reached the stage of first drafts for their section of the report. When the final drafts have been forwarded to the coordinating committee, they will be edited and the necessary introductory and concluding chapters will be added.

The Associate Director is co-editor of a Fogarty International Center monograph on Prevention of Kidney and Urinary Tract Disease which has been planned to include chapters as follows: introductory, genetic renal disorders, obstructive uropathy, glomerular diseases, urinary tract infection, interstitial nephritis, urolithiasis, acute renal failure, hypertension and renal disease, pregnancy and renal disease and evaluation of screening and health maintenance. The disease chapters will address topics such as: Introduction to include: magnitude of problem, incidence; prevalence; morbidity; mortality; surveillance; detection; treatment; control, etc., socio-economic impact justification of effort limitations: delineation of scope, sequelae of diseases, recommendations. Disease Itself - definition of disease, cause of disease, natural history of disease, and treatment (if any); Conclusions and Recommendations 1975 and 1976 - value of detection, research needed.

Cooperation with other programs involved with hematology research has included the Associate Director serving as vice chairman of the Interagency Cooley's Anemia Coordinating Committee and her working with the National Heart and Lung Institute's Division of Blood Diseases and Resources in areas of common interest.

Now in its twelfth year, the Kidney Disease and Urology Program has over 230 active research grants whose awards total $14.2 million during fiscal year 1975. Over three quarters of the research grants funded by this program support basic investigations about kidney function and its relation to disease as well as studies of the lower urinary tract. The Program's responsibilities continue to encompass the investigation of etiology, pathogenesis, diagnosis, and treatment of specific kidney and urinary tract diseases, within the relevant areas throughout all periods of life in which they may occur. The total program of research support includes studies of a very fundamental nature as they are associated with the more specific objectives, including: lupus nephritis, auto-immune processes of the kidney and urinary system; basic and clinical studies of the kidney and urinary tract in disease states such as nephritis, nephrosis, polycystic disease, diabetic nephropathy, urolithiasis, glomerulonephritis, and glomerulonephropathies; pyelonephritis and other nephritides; renal regulatory mechanisms including the renin-angiotensin-aldosterone system; all studies related to dialysis and the uremic or anephric individual; basic and applied studies on renal transplantation including techniques of organ preservation and storage; renal function, including metabolic and transport mechanisms and their disorders. There are overlapping areas of interest with other institutes within the NIH in their mutual interests in end-stage renal disease, renal function in transplantation, pyelonephritis, glomerulonephritis; calcium and phosphorus, fluid and electrolyte balance, vascular components of kidney structure and function; the paraplegic bladder and so forth.

Uremia.

End-stage kidney disease or uremia is recognized from a composite of signs and symptoms caused by the kidney's inability to remove the body's metabolic wastes and maintain the body homeostasis. One of many projects in support of the fight against end-stage kidney disease by NIAMDD is the pursuit of what is believed to be a circulating inhibitor of sodium transport (natriuretic factor), which is thought to play a role in regulating sodium excretion, both in uremia and health. The importance of the natriuretic factor is obvious and therefore much time and effort still must be exerted toward preparing the substance as a pure fraction. There are thirty other projects receiving NIAMDD support totalling better than $2.5 million that are directed towards the understanding of the cause(s), course, treatment and possible prevention of end-stage kidney disease.

Experimental Glomerulonephritis.

A number of studies underway are attempting to answer the many questions about the mechanisms responsible for the reduction of glomerular filtration rate, for proteinuria and for altered sodium handling in glomerular diseases in humans. From studies reported by one laboratory, it is apparent that patients with renal disease and proteinuria may have one of three basic types of permeability patterns:

A. A normal pattern, which is usually seen in patients with the mildest lesions. It has also been seen in patients with acute glomerulonephritis who have eventually recovered completely from their disease.

B. An increase in permeability to large molecular weight polyvinyl pyrrolidone (PVP) molecules so that molecules of a size normally excluded from the urine pass through the glomerulus and are found in the urine. In addition, the clearances of molecules usually present in the urine in small concentrations are increased, a pattern frequently seen in patients with severe glomerulonephritis.

C. A decreased clearance of small molecular weight PVP molecules, which are molecules normally present in urine with a clearance equal to that of inulin. However, in certain patients with renal disease, the clearance of these molecules never equals that of inulin, their clearance sometimes being reduced to 50% or less than that of the simultaneously determined clearance of inulin. This pattern may or may not be associated with increased permeability to larger molecular weight PVP molecules and has been seen in patients with a variety of glomerulonephritides and in idiopathic nephrotic syndrome.

Even though abnormalities in glomerular permeability have not been correlated with specific pathologic abnormalities found on renal biopsy, it is already apparent that permeability patterns may change either spontaneously during the course of a disease process or coincident with the administration of drugs. Preliminary data in animals reported by one group relating to experimental glomerulonephritis indicate that steroid therapy may correct patterns of glomerular permeability and may improve both renal function and the animal's survival.

Urinary Tract Infections.

An initial clinical and laboratory observation in which antibody-coated bacteria in the urine have proved useful as an indicator of the site of urinary tract infection has been reported by one group. These observations have been confirmed independently by others who correlated bacterial antibody-coating with the presence of upper urinary tract infection when using bladder washout techniques.

In further patient studies in this laboratory, positive bacterial antibody-coating has been found in most patients with chronic pyelonephritis or in patients with acute pyelonephritis. Twenty-two of their 76 patients who have been followed for up to 27 months to compare their course with the results of sequential examination for bacterial antibody-coating have been found to have persistent infection, relapse or reinfection. These observations suggest the persistence of antibody in chronic parenchymal infection, absence of antibody in recurrent or persistent cystitis, and the appearance of antibody with invasion of the upper urinary tract.

This group has now initiated screening studies in a Diabetes Clinic to ascertain the prevalence of urinary tract infection in a diabetic population, the incidence of parenchymal as compared to lower tract infection, and the relationship of infection on the development of diabetic nephropathy. This patient group will form the core of a long-term study of the applicability of the antibody-coating test in evaluating various therapeutic regimens. A collaborative study has been undertaken to evaluate the sequential changes in antibody-coating in women with acute pyelonephritis of pregnancy, who were free of significant bacteriuria on earlier examinations.

Laboratory studies are continuing to define more precisely the correlation of bacterial antibody-coating with urinary levels of specific immunoglobulins, and to ascertain the complement-fixing and opsonic properties of the antibody-coating the bacteria.

Transplantation.

The Program supports twenty grants which relate to kidney transplantation and which may produce information relating to greater success of kidney transplantation and/or knowledge of the immunology of kidney tranplantation. For example, a large transplantation center has been systematically screening one hundred thirty-two transplant recipients for viral infections and the findings correlated with the clinical course of the patients. One hundred ten patients showed evidence of infection with herpesviruses and eighty-nine patients showed laboratory evidence of infection with cytomegalovirus (CMV) uncomplicated by bacterial infections or technical complications. Patients without viral infections were usually asymptomatic despite the persistence of viral excretion in the urine.

In contrast, the onset of viral infections were almost always accompanied by a significant clinical illness characterized by fever, leukopenia, and renal malfunction. Of eighty-nine patients with cytomegalovirus infections, eighty-three survived at least three months. Renal biopsies demonstrated typical rejection reactions in all the biopsied patients and renal malfunction usually responded to antirejection treatment. Six of the eighty-nine patients with CMV infections died within a month of viral isolation. These patients could be distinguished from those who recovered by a decreased or absent antibody response to the virus, suppressed lymphocyte responses to mitogen in autochthonous blood, and absent histologic evidence of rejection in the renal allografts.

Thus, two paradoxical responses to CMV infections are seen in transplant patients. In the relatively immunocompetent patient, the infection is associated with renal allograft rejection, a prompt antibody response to the virus, and recovery. The severely immunosuppressed patient cannot make an antibody response and does not exhibit allograft rejection as a cause of renal malfunction. In addition, he may be further immunosuppressed by the viral infection and is susceptible to sequential opportunistic infections leading to death.

Diuretics.

Furosemide is one of the newer and widely used potent diuretics, whose main action is on the distal convoluted tubule and loop of Henle of the kidney. This drug is usually given to patients with pulmonary edema or edema due to renal failure and is thought to produce diuresis by inhibiting the action of the sodium-potassium ATPase system, thereby preventing the reabsorption of sodium and consequently water. Many clinical reports of transient and permanent deafness have been reported with the use of ethacrynic acid. Several reports of transient deafness and one case of permanent deafness have been reported following the use of furosemide. Experimental studies in animals have shown that furosemide produces morphological changes in the stria vascularis of the inner ear.

A group at the University of Minnesota noted six cases of permanent deafness encountered in a prospective study of the hearing in renal dialysis and transplant patients over a three year period, and in each case, furosemide was being administered at the time of the reported onset of the hearing loss.

In the six cases seen, extensive review of their medical history and drug administration suggested that a period of furosemide administration during impaired or critical renal function was the one factor common to each patient. The onset of deafness was usually insidious and gradually progressive up to six months after the suspected drug administration.

With the experimental evidence that furosemide does induce stria damage and the absence of other recognized causes of deafness in a series of patients, furosemide must be considered the etiological agent responsible for the permanent sensorineural hearing loss.

Lithium Effect on the Kidney.

It has been well-established that long-term lithium therapy is associated with the appearance of a defect in urinary concentrating ability. Neither the rapidity of onset of the lithium-induced defect nor its reversibility following the cessation of lithium administration have been well defined. Studies were initiated in one laboratory in the small African primate, *Galago crassicaudatus*, to evaluate the immediate and short-term effects of lithium on the urinary concentrating mechanism *in vivo*, to assess the probable site and spontaneous reversibility of any defect that might be observed, and to evaluate the extent to which any adverse effect of lithium might be prevented or minimized by pre-treatment with amiloride (an agent known to inhibit the epithelial effects of lithium *in vitro*). Water clearance ($^{C}H_2O$) was unchanged by lithium infusion whereas tubular clearance of water ($T^{C}H_2O$) reabsorption was depressed at all levels of solute excretion. Spontaneous recovery of $T^{C}H_2O$ had nearly occurred within one week after short-term lithium infusion. In addition, lithium-induced depression of $T^{C}H_2O$ reabsorption was prevented partially by pre-treatment with i.v. amiloride. The results suggest that alterations in the renal concentrating mechanism can occur rapidly following the onset of lithium administration. They also imply that impaired urinary concentration is due, at least in part, to lithium induced antagonism to the action of vasopressin on the collecting duct.

Polycystic Kidney Disease (PKD).

Polycystic kidney disease is an inherited disease whose symptoms are apparent by the fourth decade of life. The disorder progresses relentlessly to chronic renal failure which can be treated only by dialysis and/or transplantation. Cardiovascular complication, such as hypertension (found in about 50% of the patients with PKD) and stroke, are prominent features of the disorder. It has been estimated that 1 in 1000 people carry the PKD gene. The last systemic study of this disease was reported in 1957. At the time of these investigations, none of the modern techniques for diagnosis of renal disease, such as excretory urograms with tomography, radioactive isotope scans of the kidney and sophisticated renal function tests were available. The primary aim of an NIAMDD supported group at the University of Washington is to perform these diagnostic tests on patients who may carry the gene for PKD. Since this disease is inherited as an autosomal dominant, 50% of the offspring of the affected patients will carry the gene and will ultimately develop the disease. Due to the delayed onset of PKD, the diagnosis in most patients is not made until the fourth decade or later. It is anticipated that one or more of the tests in this study will allow early detection of PKD carriers. In that case, offspring of affected patients who are not carrying the gene can be relieved of the worry of certain disease; on the other hand, those patients who are found to carry the gene can benefit from genetic counselling as well as guidance with respect to planning their goals and lifestyles. Early diagnosis will also facilitate management of the patients, especially with regard to hypertension and kidney infection.

In a pilot study reported by the group involving twenty offspring of patients with documented PKD, abnormalities detected by excretory urograms were found to precede evidence of deterioration of kidney function such as urinary concentration, which is probably the first affected kidney function. Renal biopsies in fifteen individuals of those patients with negative excretory urograms are being evaluated and correlated.

Workshop on Benign Prostatic Hyperplasia.

Of the 100 scientists attending the Workshop, which was held at the NIH on February 20-21, 1975, fifteen were from outside of the United States. The program included presentations by twenty-five individuals who are experts in their respective fields, including urology, microbiology, endocrinology, anatomy, pathology or biochemistry. The intent of this meeting was to bring together outside consultants, drawing individuals from disciplines other than urology, to review, evaluate and indicate new directions for research in Benign Prostatic Hyperplasia (BPH). It was anticipated that the participation of basic scientists not involved in research of this disorder, along with researchers who were, would provide a stimulus for producing an objective evaluation of current knowledge as well as an impetus for initiating new approaches for possible future investigations. A monograph covering the presentations and relevant discussions will be published through the U.S.

Government Printing Office by late 1975 or early 1976.

International Congress.

Although the Institute was not able to honor a request made by the American Nephrology Society for a block travel grant in support of the VI International Nephrology Congress, scheduled for June 8-14, 1975 in Florence, Italy, several NIAMDD grantees received authorization to use funds from their own NIH grants to partially support their attendance and allow for the interpersonal exchange of information and ideas.

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FOR
EXTRAMURAL PROGRAM ACTIVITIES

During FY 1975, further advances were made toward the implementation of the program-cluster approach and toward the reorganization of Extramural Program Activities to meet the many requirements imposed by the transition. Among the highlights have been the appointment of an Associate Director for the Kidney-Urology-Hematology cluster and the appointment of new Associate Directors for the Digestive Diseases-Nutrition cluster and for Extramural Program Activities. For organizational and operational purposes, Program Directors from the remaining two clusters (Arthritis-Orthopedics-Dermatology and Diabetes-Endocrinology-Metabolism) have been acting as Cluster Coordinators until such time as Associate Directors are appointed. The Cluster Associate Directors and Coordinators have been meeting on a regular basis with the Associate Director for Extramural Program Activities. As an operating group, their purpose has been to provide coordination to activities across the Extramural Programs, as well as within the clusters and programs. In addition, they have been serving as a panel for the review of requests for personnel actions.

At the operational level, implementation of the cluster concept has been augmented and enhanced by the initiation of a first step towards a team approach to the administration of extramural programs. In particular, individual Grants Management Specialists have been assigned responsibilities with respect to each of the clusters. The objective has been to develop an administrative capability which will benefit from direct interaction between scientific and management staff.

In FY 1975, pre-Council meetings for the joint review of applications by Program Staff and Extramural Program Activities staff were initiated. The purposes of these preparatory meetings have been to expedite the proceedings at Council and the processing of applications and awards following Council, to identify those applications requiring special Council consideration or staff consideration, and to prepare and provide advice and recommendations from staff for Council.

The cluster concept has also had its impact on the National Arthritis, Metabolism, and Digestive Diseases Advisory Council. For the past two years, members of the Council from the Digestive Diseases and Nutrition research communities have been convening, immediately prior to each Council meeting, to review the various applications in their areas and to discuss programmatic concerns. At the March 1975 Council meeting it was decided to extend this approach to the remaining three clusters, beginning with the June 1975 meeting. As a result, Council panels have been constituted for each of the clusters on a temporary basis; this innovation will be evaluated by the Council after it has been tried out for a few rounds.

Over the year, the objectives of the Office of the Associate Director for Extramural Program Activities have been steadily evolving and expanding in the directions of (1) defining, programming, and planning for improved utilization of research-support mechanisms (e.g., Center Grants, contracts);

(2) improving program development, evaluation, and planning; and (3) enhancing communication with the multifaceted multidisciplined, NIAMDD research community. As an example of the latter, Extramural Program Activities staff have joined with Program staff in developing, planning for, and staffing of an informational suite at the Annual Meeting of the American Rheumatism Association. Plans are underway for making staff and facilities available for other meetings which large numbers of applicants, grantees, and potential applicants attend. The Associate Director has also been directly involved in many activities related to the Diabetes Commission and to the Arthritis Commission. A most important role has been in providing an advisory function to a committee of the Diabetes Commission which is looking into the development of the Center-grant program.

Implementation of the objectives of the Office of the Associate Director for Extramural Program Activities has been augmented by the addition of two staff members. One joined the Institute in February as Special Assistant to the Associate Director. The second came to the Institute in May bringing a particular expertise in the technology and science of program development and evaluation.

In serving a coordinative function for the Extramural Program clusters, one of the major objectives has been to decentralize some of the functions previously contained within the Office of the Associate Director for Extramural Program Activities. It was felt that program interest might be better served by having many decisions made at that level, particularly with respect to the programmatic assignment of applications and with respect to their funding of the various grant programs. However, for purposes of coordination and efficiency, liaison with other Bureaus, Institutes, and Divisions is still maintained through this Office.

Processing of Grant Applications

Serious strains were placed on the entire Extramural Programs area by the relatively large number of applications which required processing during the final third of the fiscal year. There was an exceptional number of applications to June Council alone, with virtually all grant mechanisms represented (i.e., fellowships, development programs, training programs, program projects, centers, and regular research grant applications). The volume for this one review round was greater than what has been the case for some entire recent fiscal years. To complicate matters further, staff were constrained to deal with a somewhat meager budget for three quarters of the year, and to accommodate to the release of a much larger appropriation at the beginning of the last quarter. Implementation of the National Research Service Act was also not without its problems: Fellowships could first be funded in late May. In addition to usual duties associated with the June Council review cycle, program staff served as Executive Secretaries of *ad hoc* training review committees for DRG in early May. Other Extramural Programs staff were directly involved in the preparation of budgets and of summary statements for these reviews. Then too, plans for the phase-out funding of the "old" (T01) training program could not be finalized and implemented until the review of the applications for the "new" (T32) training program was completed, and until budgetary matters were clarified.

Grants Management Branch

During FY 1975, the team concept of joint administration by management and scientific staff was partially implemented with respect to the handling and processing of applications and awards. That is, Grants Management Specialists were assigned much closer working relationships and responsibilities with respect to the various clusters. To date, these assignments have been limited to the competing applications and it is anticipated that in the future each Grants Management Specialist will collaborate with program staff in the processing of all applications. Another operational aspect of this new approach is that Council letters for those applications that were approved and are to be funded, are now prepared for the signatures of both Program and Grants Management officials.

On the fiscal side, the change from budgeting-by-mechanism to budgeting-by-program has had a great impact on the Branch. This has required keeping track of encumbrances on individual program and cluster bases, and the change has resulted in the need for maintaining 30 separate accounts, rather than just 3 as in past years.

Again this year, as has been the case in most recent years, there has been an up and down struggle with budget levels. In particular, the Institute was required to operate at the levels necessitated by the rescission which was proposed by the Executive for the FY 1975 budget. Although this proposal was rejected by the Congress, its implementation had the effect of drastically slowing the encumbering process for several months. Once the rescission was rejected, the operations of the Extramural Programs had to accommodate to a shift from a level of approximately $108 million to the level of the approximately $135 million in the Appropriation Bill. This had the effect of again compacting the time available within the year to process the bulk of the competing awards.

Administrative Services

The number of personnel actions processed by Administrative Services increased considerably during FY 1975. There were 17 such actions in FY 1974 and approximately 50 in FY 1975. As changes in staff continue, and if the availability of "slots" increases and restrictions on promotions are reduced, it is anticipated that the personnel function will continue to occupy considerable attention in this Office.

Of the various functions performed by the Administrative Office, the one which has shown the largest increase, and which has occupied the greatest amount of staff time, has been the administration and processing of travel requests. The establishment of the Diabetes and Arthritis Commissions and of the ad hoc training review committees, together with an increase in staff travel and reactivation of the Chairman's Grant, has resulted in a virtual trippling of the administrative work in this area. This has been especially the case during the last half of the fiscal year.

Staffing

The Extramural Program areas of the Institute are in a state of serious understaffing. When the workload has been particularly heavy (as has been the case this past year, and as promises to continue to be the case in the near future), we have all become especially sensitive to the seriousness of the needs. Yet, the performance of all staff, during the recent year of transition and change, has been most admirable, particularly with respect to meeting established objectives and deadlines. To say that there is a great need for technical and secretarial help in the Data Processing and Grants Management Branches in particular, and in Administrative Services, would be an understatement! In addition, the ratios of Scientific Programs staff to the numbers of applications, awards, dollars, and grantees, is at a level which is seriously low enough as to potentially have a direct effect on the quality of program administration.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

SCIENTIFIC COMMUNICATIONS SECTION

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The Scientific Communication Section has responsibility for general programming and planning of communication activities concerned with various scientific areas charged to NIAMDD. The present activities can be generally grouped into four main categories.

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of motion pictures, thesaurus development, etc.

Current awareness literature services are provided by the Institute in several specialized areas. At present, there are four publications with hierarchically arranged listings of literature citations and one publication which includes abstracts. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its eighth volume, is intended to provide selected abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical. Additionally, complete coverage of four selected gastroenterological areas is being performed and compiled into formatted magnetic tapes, suitable for searching through the facilities at DCRT. This additional information, along with the regular issues of GASTROENTEROLOGY ABSTRACTS AND CITATIONS, are being utilized by the Digestive Diseases and Nutrition Program of the NIAMDD. Four of the publications, ARTIFICIAL KIDNEY BIBLIOGRAPHY (eighth volume), ENDOCRINOLOGY INDEX (eighth volume), DIABETES LITERATURE INDEX (tenth volume), and INDEX OF DERMATOLOGY (seventh volume) contain citations to current world-wide literature which have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS).

To produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects, we have cooperated with the National Library of Medicine, and in one case also with the University of Minnesota and the University of Rochester.

The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching and to those who are interested in these highly specific areas.

Outstanding scientists working in the field covered by the publication have been assembled for each publication as editorial advisors to ensure the continuation of the high quality of these publications.

Expansion of the ARTIFICIAL KIDNEY BIBLIOGRAPHY is progressing with the inclusion of kidney diseases, nephrology, and urology. The first and second experimental issues were utilized by the editorial advisors in recommending revisions for a third experimental issue. The third experimental issue is in preparation, and work with the National Library of Medicine's Medical Subject Headings Section is on-going to include kidney-related vocabulary in the National Library of Medicine's computerized vocabulary. Clearance to publish has been received from the Office of Management and Budget to begin the new KIDNEY DISEASE AND NEPHROLOGY INDEX as a recurring bibliography.

Conferences and workshops which are sponsored by NIAMDD are planned and organized by the Scientific Communications Section. In addition to the organization, which usually includes physical arrangement as well as program planning, with the conference chairman the publication of the proceedings also falls under the auspices of the Scientific Communications Section.

This year nine conferences and workshops, sponsored either completely or jointly, were the Eighth Annual Contractors' Conference, Workshop on Benign Prostatic Hyperplasia, Workshop on Research in Scoliosis, Second International Congress on Immunology, Conference on Gastrointestinal Aspects of Uremia, organizational workshops of the National Commission on Diabetes, the Diabetes Mellitus Coordinating Committee, and the National Commission on Arthritis, and the second meeting of the National Commission on Arthritis.

The National Cystic Fibrosis Research Foundation is compiling a comprehensive bibliography with indexes which will cover various aspects of cystic fibrosis, and will be available in the future.

National Library of Medicine's on-line MEDLARS (Medical Literature Analysis and Retrieval System) is available to NIAMDD staff through NIAMDD's call number. The Scientific Communications Office is responsible for coordinating this use and is a reference source for the NIAMDD staff.

Project Title: ARTIFICIAL KIDNEY BIBLIOGRAPHY

Professional Personnel: Mrs. Billie B. Mackey
Ms. Joan W. Chase

Man Years:

Total:	0.9
Professional:	0.6
Other:	0.3

Project Description:

The quarterly recurring bibliography, ARTIFICIAL KIDNEY BIBLIOGRAPHY, is a bibliography on kidney failure, artificial kidneys and kidney transplantation and is produced with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS) by the Scientific Communications Section, NIAMDD. Each issue contains approximately 500 citations with the citations arranged hierarchically to group similar subjects together. An author index is included in each issue. A listing of annual and final technical reports submitted to the Artificial Kidney-Chronic Uremia contract program are included. These reports are available through the National Technical Information Service for a fee.

The ARTIFICIAL KIDNEY BIBLIOGRAPHY is available to National Institutes of Health grantees or contractors with interests in artificial kidneys, government agencies with responsibilities in the area, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Expansion of this bibliography with the inclusion of kidney diseases, nephrology and urology is progressing. The first and second experimental issues were utilized by the editorial advisors in recommending revisions for a third experimental issue. The third experimental issue is in preparation, and work with the National Library of Medicine's Medical Subject Headings Section is on-going to include kidney-related vocabulary in the National Library of Medicine's computerized vocabulary. Clearance to publish has been received from the Office of Management and Budget to begin the new KIDNEY DISEASE AND NEPHROLOGY INDEX as a recurring bibliography.

Project Title: DIABETES LITERATURE INDEX

Contract Number: PH43-67-663

Amount: $96,117

Contractor: University of Minnesota

Project Officer: Mrs. Billie B. Mackey
Ms. Joan W. Chase

Man Years:
Total: .52
Professional: .32
Other: .20

Project Description:

A monthly current-awareness publication, the DIABETES LITERATURE INDEX, is now in its tenth volume. This bibliography was brought about through the cooperative efforts of the Institute, the National Library of Medicine, and the American Diabetes Association, and three universities, (University of Minnesota, University of Rochester, and Case Western Reserve University), and many individual scientists.

MEDLARS magnetic tapes containing all of the current biomedical literature citations used for the preparation of NLM's INDEX MEDICUS, are used to retrieve all diabetes-related bibliographic citations monthly. Until 1971 the DIABETES LITERATURE INDEX consisting of a keyword in title index and an author index resulted from a computer printout. From 1971 to date, the magnetic tape version of these indexes is prepared at the University of Minnesota and is used to generate copy for offset printing by the Government Printing Office on Linotron. This monthly publication consists of three formats, a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes literature, an author index including index terms with the citations (for the first author). Grouping the citations under a preconceived hierarchical structure, with related subjects together, makes it easier for users to scan their particular interests in one section.

DIABETES LITERATURE INDEX is published monthly by the Office of Scientific Communications, NIAMDD, and is available to National Institutes of Health grantees or contractors with interests in diabetes, government agencies with responsibilities in this area, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title:	ENDOCRINOLOGY INDEX
Professional Personnel:	Mrs. Billie B. Mackey
Man Years:	Total: .7 Professional: .4 Others: .3

Project Description:

A bimonthly recurring bibliography, the ENDOCRINOLOGY INDEX, is now in its eighth volume. Each issue contains approximately 4,000 citations of the current world literature in endocrinology. Citations are grouped in eight categories (pituitary, thyroid, etc.), and each category has a preconceived hierarchical organization of subject headings. Index terms, assigned to the original article by the indexer, appears in the author section, and author and subject indexes appear in each issue. This bibliography is produced, with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS), by the Scientific Communications Section, NIAMDD.

Citations from other sources are selectively added, including citations of books of potential interest to endocrinologists which have been chosen from National Library of Medicine's Current Catalog as well as citations of books and articles of potential interest to neuroendocrinologists which have been chosen from the five publications of the Brain Information Service. Work with the Medical Subject Headings Section of the National Library of Medicine for inclusion of endocrine-related vocabulary in the computerized vocabulary is on-going.

ENDOCRINOLOGY INDEX is available to National Institutes of Health grantees or contractors with interests in the field of endocrinology, government agencies with responsibilities in the field, and medical school libraries. It is for sale by the Superintendent of Documents, Government Printing Office for a fee. The free mailing list was circularized to insure currency.

Project Title:	*Gastroenterology Abstracts and Citations*
Contract Number:	NIH 73-2227
Amount:	$90.019
Contractor:	Franklin Institute
Project Officer:	Ms. Joan Chase
Other Professional Personnel:	Dr. James Burks Dr. Barry Kemler Mrs. Billie B. Mackey
Man Years:	Total: .25 Professional: .1 Other: .15

Project Description:

In January 1966 the NIAMDD began publication of a current-awareness journal for gastroenterologists. *Gastroenterology Abstracts and Citations*, now in its eighth volume, appears monthly. Each issue contains approximately 350 abstracts of the most significant gastroenterological literature published in the world and approximately 750 citations covering other relevant gastroenterological literature. The abstracts and citations are divided into three main categories--Pre-clinical Sciences, Diagnostic Procedures, and Gastrointestinal Diseases. These categories are subdivided into more specific groupings. Each issue contains a subject and author index and these are cumulated annually.

Since 1973, in addition, the contractor has been furnishing complete literature coverage on four selected areas, formatted on tape suitable for searching, for the benefit of the Digestive Diseases and Nutrition Program of the NIAMDD. During this fiscal year, the data base was tested for type and depth of indexing ease of retrieval and ability to access through DCRT programs. A report on the findings is being prepared.

Gastroenterology Abstracts and Citations is available, free upon request, to qualified investigators with NIH grants or contracts in the field of gastroenterology, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, U.S. Government Printing Office.

Project Title:	Index of Dermatology
Professional Personnel:	Ms. Joan Chase Mrs. Billie B. Mackey
Man Years:	Total: 0.3 Professional: 0.2 Other: 0.1

Project Description:

To implement the NIAMDD Dermatology Program as well as respond to the increased interest and activity in dermatology, NIAMDD undertook the publishing of Index of Dermatology in April 1972.

The Index of Dermatology is a monthly recurring bibliography on skin diseases, both general and specific, with aspects such as immunology, mycology, virology, and oral disease. The Index which is being produced monthly with the aid of the National Library of Medicine's MEDLARS (Medical Literature Analysis and Retrieval System) by the Scientific Communications Office, NIAMDD, contains about 1,200 citations per issue. The citations are arranged hierarchically to enable similar subjects to appear together, and an author index is included in each issue.

Fourteen outstanding scientists working in the area of dermatology have been assembled as editorial advisors to this publication. In December of 1974, the editorial advisors met in Chicago to discuss changes and improvements to the Index. It was suggested at that time that venereal disease terms and the Current Catalog of NLM be added.

The Index of Dermatology is available free to National Institutes of Health grantees or contractors who are involved in work related to dermatology, to other government agencies with responsibilites in the area, and to medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office, at a nominal fee. The mailing list was circularized to insure currency.

Project Title:	Artificial Kidney - Chronic Uremia Program Eighth Annual Contractors' Conference and Proceedings
Professional Personnel:	Dr. Robert J. Wineman Dr. Fernando Villarroel Mrs. Billie Mackey Ms. Joan Chase
Man Years:	Total: 0.4 Professional: 0.2 Others: 0.2

Project Description:

The Eighth Annual Contractors' Conference was held January 13-15, 1975. About 175 participants who were contractors and their staff, consultants to the Artificial Kidney - Chronic Uremia Program, and Institute staff attended. The sessions were devoted to reports of existing contract-funded research and development projects. The presentations were grouped in three sessions: 1) Biochemistry and Pathophysiology; 2) Biocompatibility, Membranes and Blood Access; and 3) Therapy, Evaluation, and Device Development. The conferees communicated research results in the main areas of the Artificial Kidney - Chronic Uremia Program. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 60 currently active research and development projects, the Program's consultants and its staff. This and future conferences will assist in attaining the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The proceedings of the Conference will be published and distributed in Summer 1975.

Project Title:	Proceedings on Adequacy of Dialysis III
Contract Number:	NIH-74-C-365
Amount:	$30,225
Contractor:	Franklin Hospital
Professional Personnel:	Dr. Robert J. Wineman Mrs. Billie B. Mackey
Man Years:	Total: 0.30 Professional: 0.15 Others: 0.15

Project Description:

This conference was held in Monterey, California, March 20-22, 1974. The purpose for the conference was described in last year's Annual Report and the Proceedings reflect the main points: 1) That since there are more than 12,000 individuals being maintained by hemodialysis there is a necessity to provide clinicians with reasonable guidelines for therapy; 2) Another objective of the conference was to explore and propose quantitative parameters of treatment to extend to dialysis patients a maximal degree of near-normal physiologic existence and rehabilitation; and 3) Where existing data were inadequate, a third purpose of the conference was to define protocols for the study of end-stage uremic lesions of the dialyzed patient and variables of patient therapy such that quantitative relationships can be established. These objectives were reflected in the Proceedings.

The Proceedings of the conference was published in Kidney International, Supplement No. 2, January 1975.

Project Title: Diet Guide for Patients on Chronic Dialysis

Personnel: Dr. Benjamin T. Burton
Mrs. Billie Mackey

Project Description:

The kidneys regulate the amount of many substances contained in the body such as sodium, potassium, and fluid. They also excrete waste products--namely end products of protein, one of the chief concerns in kidney disease. When the kidneys lose the ability to function properly, these materials begin to increase in the blood to toxic amounts. Therefore, it is necessary to regulate the amount of these substances taken in by the food eaten by a patient on chronic dialysis. This is done by placing the artificial kidney patient on a special diet. This booklet guides the patient and makes it easier for him to follow the diet which has been prescribed for him. All of the foods allowed on the diet are divided into groups arranged in the form of exchange lists. The lists show the protein, sodium and potassium content of the foods and the amounts to use for one exchange.

Project Title: Evaluation and Planning Workshop on Idiopathic Scoliosis

Professional Personnel: Dr. Ronald Lamont-Havers
Mrs. Billie B. Mackey

Project Description:

The workshop was held November 14-16 in Chicago, Illinois, and among the objectives were (1) to identify areas in which investigation would be of major importance to the better understanding, prevention and treatment of scoliosis, establish priorities and suggest a mechanism for initiating the recommended investigation, (2) to develop proposals to determine the magnitude of scoliosis, identify patients and establish therapeutic programs, (3) to recommend methods the Scoliosis Research Society can utilize in the implementation of research and education, and (4) to fund recommended programs.

Members of the workshop were divided into five committees dealing with the etiology, pathophysiology (biomechanics, bracing, instrumentation), pathophysiology (cardiopulmonary, metabolic) and sources and mechanisms of funding. Recommendations included (1) correlation of clinical aspects of growth and development with the radiographic determination of skeletal age and serum hormone levels, and the study of connective tissues and genetics; (2) routine school screening examinations and educational programs; (3) a definition of scoliosis for a reporting system and data bank establishment, and (5) development of means of research funding by the Society.

Project Title: Proceedings on End-stage Diabetic Nephropathy

Amount: $7,300

Contractor: Minneapolis Medical Research Foundation

Professional Personnel: Dr. Robert J. Wineman
Mrs. Billie B. Mackey

Man Years: Total: 0.02
Professional 0.02
Other: Negligible

Project Description:

The conference was held in Minneapolis, May 23-24, 1974.

This conference was designed to examine the problems associated with treating end-stage diabetic nephropathy patients and to disseminate the information that has been gained by the few U.S. centers which have been working with diabetic patients. The purpose was so that involved clinicians would be better able to make rational, informed decisions in managing this problem.

Consequently, proceedings of the conference were published in *Kidney International*, Supplement No. 1, October 1974, for distribution to those interested.

Project Title: Proceedings--International Conference on Bone Mineral Measurement

Professional Personnel: Dr. G. Donald Whedon
Mrs. Billie Mackey

Project Description:

These proceedings are the second of a series on the subject of bone measurement sponsored by the University of Wisconsin Bone Mineral Laboratory. The first proceedings, Methods of Bone Mineral Measurement, reported on the meeting of May 22-23, 1970. These proceedings describe a similar meeting held on October 12-13, 1973, at the Regency Hyatt House near the Chicago O'Hare Airport.

The meeting was attended by 140 participants of whom about half were physicians and the rest basic scientists. The communication between the various disciplines was excellent. Some of the discussion was captured and is included after each paper.

In the past decade the measurement of bone mineral content by photon absorption has evolved into an accepted research procedure as well as a tool for clinical and biomedical investigations. This development has not been without problems, and we see in meetings like these the difficulties still with us concerning calibration, choice of instrumentation and measuring sites, and the interpretation of results to mention but a few. At the same time there are new developments, such as the use of dual photon absorption, Compton scattering and neutron activation. It is only through meetings like these that the problems and concerns of both physical and biomedical researchers can be expressed. It is our hope that continuation of this communication Proceedings--International Conference on Bone Mineral Measurement, among interested researchers will facilitate resolution of these problems, and aid in the incorporation of new perspectives.

The proceedings have been published in monograph form by the U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, National Institute of Arthritis, Metabolism, and Digestive Diseases, Publication No. (NIH) 75-683.

Project Title: Proceedings of the Symposium on the Endocrine Functions of the Kidney

Personnel: Dr. Benjamin T. Burton
Mrs. Billie Mackey

Project Description:

These papers were presented at the First Rogosin Kidney Center Seminar--Endocrine Functions of the Kidney, May 10, 1974. The New York Hospital - Cornell Medical Center and Artificial Kidney - Chronic Uremia Program, National Institute of Arthritis, Metabolism, and Digestive Diseases were co-sponsors.

The major stimulus for the symposium was the remarkable advances that have been made in treating patients who no longer have enough renal excretory function to maintain life. The treatment of kidney disease is apparently one of the major success stories of the past twenty years. The most recent figures released by the National Center for Health Statistics indicate a 77% drop in deaths from the major kidney diseases. The causes for this statistical event are certainly not immediately obvious; the results, though, have led to new investigative approaches in medicine, founded on the precepts of classic physiology. This symposium represents an approach to the study and treatment of patients with end-stage kidney disease in terms of the metabolic and endocrine functions of the kidney.

The proceedings were published in The American Journal of Medicine, Vol. 58, No. 1, January 1975.

Project Title: Workshop on Benign Prostatic Hyperplasia

Professional Personnel: Dr. Nancy B. Cummings
Dr. M. J. Scherbenske
Mrs. Billie B. Mackey

Project Description:

This workshop was held in Bethesda, Maryland, February 20-21, 1975. It is well known that Benign Prostatic Hyperplasia (BPH) occurs in fifty percent of all males past the age of 50, which results in many physical problems, including chronic urinary tract infection, renal insufficiency, bladder dysfunction, calculus disease, hypertension and significant morbidity and mortality due to the surgery necessary to correct the disease. The etiology of this second growth of the prostate is unknown and at this time its only satisfactory treatment is surgical removal.

A thorough review of the research underway in this important field served as a primary stimulus to plan a workshop, which would in turn provide an up-to-date summary of the rapidly increasing body of knowledge relating to BPH. Consultants in this area of investigation express the universal opinion that such a summary could be a real contribution with the increasing interest in and potential for understanding BPH.

A limited number of leading investigators in various disciplines related to this very important but neglected area of research came together for the workshop. A monograph covering the proceedings will be published in the future.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM

Summary Report

Introduction

The Annual Reports for the previous years provide detailed background on the problem of end-stage kidney disease and elaborate on developments in the modality for its treatment, chronic intermittent hemodialysis. In addition, these reports give a detailed description of the planned and centrally directed contract program of research and development organized at the National Institute of Arthritis and Metabolic Diseases in 1966--the Artificial Kidney-Chronic Uremia Program--which is aimed at development of improved and less expensive dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. This background material is not repeated here but the reader might wish to familiarize himself with it by perusing the previous reports.

Current Activities

The Artificial Kidney-Chronic Uremia Program endeavors to bring about improvements in artificial kidney apparatus and methodologies, to increase their effectiveness, to decrease their initial and operational cost, and to improve the rehabilitation of patients treated with chronic intermittent dialysis. Optimal development of new, better dialysis apparatus and methods is still hampered by incomplete knowledge concerning the specifics of the uremic syndrome. Hence, knowledge gained from research into the fundamental aspect of the toxic nature of uremia and basic and clinical studies into the long-term effects of uremia and of chronic intermittent dialysis are critical to arriving at a reasonable solution to the overall problem. Thus, the Program not only sponsors hardware improvement per se, but stimulates and supports a wide spectrum of research ranging from fundamental studies designed to clarify the toxic nature of uremia and clinical studies on the complications of chronic uremia and maintenance dialysis, to development of improved dialysis or apparatus. In recent years there has been an increasing emphasis on laboratory and clinical studies intended to bring about a greater understanding of the nature of uremia and its major complications.

At present about 70 research and development projects are supported by contracts with universities, nonprofit and industrial research laboratories to give a broad approach to problems of highest priority. Projects are focused on problem areas including clinical trials of more efficient or less costly dialyzers, development of special adsorbents for uremic toxins, studies to elucidate the nature of the aberrant metabolic changes in uremia, and the maintenance of a national registry of all patients who are being maintained with the aid of dialysis. The Program is also funding studies aimed at isolation and identification of toxic factors which must be removed from the patient's blood, in addition to metabolic wastes and other substances known to accumulate during kidney failure. A large number of research projects is also devoted to the pathophysiology of uremia in an effort to prevent or control complications in patients who have lost their kidney function and are being maintained with the aid of artificial kidneys. Much of the future success in simplifying the treatment of patients with end-stage kidney disease depends on developments in these and other areas of fundamental research.

Accomplishments

New Ultrafiltration Cell

Research contractors supported by the Artificial Kidney Program recently developed an ultrafiltration cell to remove excess sodium and water efficiently, rapidly, and inexpensively from fluid-overloaded patients on chronic hemodialysis. The device, which can be used in series with an artificial kidney or as a separate unit, may enable physicians to treat more rapidly and efficiently patients with edema secondary to cardiac failure with acute pulmonary edema. Current treatment of edema, regardless of its cause, employs low-sodium diets and diuretic medication. Treatment usually requires days to weeks, while a few unresponsive patients may require salt and water removal with peritoneal dialysis or the artificial kidney.

In treatment with the new device, arterial blood is delivered to the ultrafilter by a blood pump, filtered and measured and returned to the patient. This therapy is simpler and safer than fluid removal by peritoneal dialysis or hemodialysis. No serious complications, such as clotting (which is prevented through use of an anticoagulant) occurred in over 100 trials. The investigators suggest this therapy could logically be extended to patients with chronic congestive heart failure or pulmonary edema, and would conceivably replace costly and prolonged hospitalization with one or two relatively brief ultrafiltration treatments.

Accelerated Atherosclerosis in Prolonged Maintenance Hemodialysis

Evidence has been accumulating in recent years to suggest that cardiovascular disease is the leading cause of death among patients undergoing chronic hemodialysis therapy. Investigators supported by the Artificial Kidney Program have obtained evidence in a retrospective study of 39 patients on prolonged hemodialysis that accelerated atherosclerosis in a major risk. The study revealed a mortality rate of 59 percent, with 14 of 23 deaths resulting from atherosclerotic complications; myocardial infarction was responsible for eight deaths, strokes for three, and refractory congestive heart failure for three. The incidence of these complications was many times higher than for non-uremic, normal and hypertensive groups of comparable age, and similar to rates found in Type II hyperlipoproteinemia (an inherited condition of having excess lipoproteins in the blood which characteristically lead to early artheroscleroses and cardio vascular impairment). The data suggest that the probability of dying from such complications increases with time on dialysis.

Abnormalities of Lipid Metabolism in Hemodialysis Patients

As part of its program to determine the causes of accelerated atherogenesis in chronic uremic patients, the Artificial Kidney-Chronic Uremia Program recently expanded its efforts in the study of abnormalities of both carbohydrate and lipid metabolism of this patient group. Some of the early findings eminating from these studies are the following: Cholesterol concentrations in patients on dialysis appear to be within normal distribution range. Plasma triglyceride concentrations are elevated in uremic patients both dialyzed and undialyzed. Very low density lipoprotein factors, which are elevated in uremic patients, present a pattern resembling Type Four Hyperlipoproteinemia. In this fraction, triglycerides and cholesterol concentrations are significantly higher than normal. In the low density lipoprotein fraction, however, uremic patients have more triglyceride and less cholesterol than normals and Type IV hyperlipidemic controls. Plasma high density lipoproteins are depressed in nephrotic patients while low density lipoproteins are elevated. The possibility that lower than normal concentrations of high density lipoproteins inhibit lipoprotein clearance is also under investigation. Hyperlipidemia in a dialysis patient frequently persists following renal transplantation. Additional studies of both patients and uremic animal models are underway directed to a more complete understanding of these phenomena and their possible relationship to accelerated atherogenesis.

Trace Element Abnormalities in Dialysis Patients

One of the possible causes of altered metabolism in the long term dialysis patient is that the individual may be presented with abnormal body burdens of various elements. To develop additional information relative to this question the Artificial Kidney Program has sponsored several new studies. Preliminary findings show that in certain dialysis populations abnormally high tissue concentrations of the following elements can be developed: strontium, molybdinum, and tin. In contrast in the same population concentrations of bromine and rubidium were significantly below normal. Further studies are planned to develop a more general data base.

New Developments in Altered Dialysis Therapy

In one of the studies of altered dialysis therapy, the investigators have found that slower flow rates of dialysate fluid have led to some unexpected patient advantages. One of these is that the motor nerve conduction velocity of the experimental group increased to the normal range. Uremic neuropathy is frequently a complication experienced by dialysis patients and is characterized by a slowing of motor nerve conduction times. A second advantageous result is that the serum cholesterol of the patients in the experimental group was appreciably reduced. Further investigation of these initial findings, as well as other alterations in dialytic therapy are in progress. One aspect of dialysis possibly related to accelerated atherogenesis is the high acetate load experienced by these patients. Acetate is a constituent of most dialysate fluids, and in the experimental therapy described above the patients received appreciably lower acetate loads.

More detailed studies of the influence of acetate on the metabolism of dialysis patients has been started.

Improvement of Assay Method for Red Cell Stimulation Factor

A continuing important problem is that dialysis patients suffer from a low concentration of red cells in their blood. At present mechanisms which cause this condition of uremic anemia, or low hematocrit, are not understood. One critical need in order to study this problem has been the need for a sensitive, accurate assay for erythropoietin, the plasma factor which stimulates red cell production. Preliminary results of one contractor of the Artificial Kidney Program show that he has succeeded in developing such an assay employing radioimmunoassay methods. This improvement in the specificity of the assay method should provide a useful tool to study the role of erythropoietin deficiency in the anemia of renal failure.

National Dialysis Registry.

The National Dialysis Registry, organized and maintained by the Institute, shows that in the United States about 15,000 patients are kept alive with the aid of artificial kidneys. The annual mortality of those on chronic dialysis is approximately 10% during the first year and from 7 to 9% during subsequent years. The Registry data indicate that the most frequent cause of death appears to be accelerated degeneration of the blood vessels akin to the accelerated atherosclerosis found in the large blood vessels of diabetic patients. Over 60% of long-term dialysis patients die of heart disease and strokes. In the coming fiscal year a new registry for End Stage Kidney will be started by the Bureau of Quality Assurance. Relevant data from the National Dialysis Registry will be transferred to the new registry, after which the present registry will be discontinued.

Adequacy of Dialysis Workshop Proceedings

Social Security Act Amendments extended MEDICARE coverage to provide dialysis when needed for about 90% of all workers and their dependents in the United States. Dialysis facilities and trained technicians will be required to treat an estimated dialysis population of 40,000 within a few years. More important will be the need to improve the physical and mental well-being of these patients to the point where they can lead normal and productive lives. Therefore, the dialysis treatment must not only save lives, but also raise the "quality of life" of the patients' remaining years.

Determining the quality of care of dialysis patients is a very difficult and complex problem. At present there is no widely accepted criteria that can be adequately measured to assess patient care, therefore the assessment of patients depends upon the physicians' impressions of each patient's sense of well-being, his ability to lead a productive life, and minimal uremic symptoms. Complicating the problem are the different treatment regimens and different equipment used with no agreement on an optimal treatment by the different dialysis centers. To aid in the search for uniform criteria, the Institute sponsored a preliminary conference in the Spring of 1972. Early in 1974 a large conference was held to assess dialysis and propose parameters of treatment to offer dialysis patients a degree of near-normal physiologic existence and rehabilitation.

Two hundred leaders and experts in the dialysis field participated in this workshop and the results were a conference proceedings published as a journal supplement, Adequacy of Dialysis.

Conferences, Publications, and Scientific Communication

The Institute regularly organizes symposia and workshops on critical problems in kidney disease research and urologic conditions. The most recent of these was a benign prostatic hyperplasia workshop in early 1975. Each year the Institute brings together the research contractors of the Artificial Kidney Program with staff and consultants for a three day presentation and discussion of all ongoing work by the contractors. Illustrated proceedings, describing the most recent advances, are published and distributed widely.

Other conference proceedings published include a symposium on the optimal treatment of diabetic patients with kidney disease, Endstage Diabetic Nephropathy and a conference proceedings, Endocrine Functions of the Kidney.

Outlook for Kidney Disease and Research and Development

Research conducted and supported by the National Institute of Arthritis, Metabolism, and Digestive Diseases has added much to the understanding and treatment of kidney disease. Clearly this review of investigation and progress in the broad area of kidney disease, artificial kidneys, and renal transplantation is not all-encompassing. Nonetheless, the variety and fruitfulness of the Institute's efforts and the stimuli to further investigation are apparent. Investigations such as those discussed in this report have increased our ability to cope with the problem of kidney failure. Continued research efforts are greatly needed. The effort to reduce kidney disease must include all of the available components - research, prevention, treatment, and education. The total program is aimed at all the primary kidney diseases as well as at end-stage kidney failure. Combined with fundamental research, which is the essential underpinning, this program should result in maximal benefits in the saving of lives and prevention of disease.

CONTRACTOR: Baylor College of Medicine

AMOUNT: $ 80,000

TITLE: Abnormalities of Carbohydrate Metabolism in Chronic Uremia

OBJECTIVES: The investigators are examining the role of skeletal muscle in the carbohydrate intolerance of uremia.

MAJOR FINDINGS: They have obtained preliminary data on glucose and alanine kinetics and interrelationships in normal and uremic patients. The values obtained by isotope method are in good agreement with reported values obtained by the hepatic vein catheter technique.

PROPOSED COURSE: Work will continue in the area of investigating the role of skeletal muscle in carbohydrate abnormalities in uremia, and will also include studies of glucose kinetics during dialysis.

CONTRACTOR: Boston University School of Medicine

AMOUNT: $ 75,914

TITLE: Lipid Abnormalities in Chronic Renal Failure

OBJECTIVE: To understand the lipid abnormalities that occur in chronic renal failure and to provide a basis for the treatment of these complications.

MAJOR FINDINGS: Fatty acid synthesis in the uremic rat is depressed: rats with a mean blood urea nitrogen level of 140± 7 mg/dl have only 20% the rate of hepatic fatty acid synthesis as did the sham operated rats.

PROPOSED COURSE: Using the in vitro perfused liver model rates of lipogenesis, glyceolysis, gluconeogenesis and ketogenesis will be measured and correlations made with dietary cholesterol and protein intake.

Contractor: University of California

Amount: $50,000

Title: Trace Element Abnormalities in Chronic Uremia

Objective:

To determine the levels of concentration of trace elements in dialyzed uremic patients; to determine the clinical importance of trace element abnormalities, and to establish the concentration of trace elements required in the dialysate solution.

Major Findings:

Techniques for collection of samples and processing for analysis have been standardized. Preliminary data has been obtained with a control population.

Proposed Course:

Termination.

CONTRACTOR: University of California at Los Angeles

AMOUNT: $105,000

TITLE: Controlled Evaluation of Maintenance Peritoneal Dialysis

OBJECTIVE: The study has two objectives: (1) To evaluate the severity and causes of malnutrition and wasting in patients undergoing long-term peritoneal dialysis, it is proposed to evaluate nutritional status in a controlled study of uremic patients randomly assigned to treatment with either hemodialysis or peritoneal dialysis. The patients will be participating in an independently funded study funded by the Veterans Administration. (2) To evaluate the relative clinical outcomes of diabetic patients assigned either to hemodialysis or peritoneal dialysis, it is proposed that prospective, controlled comparison of home peritoneal and home hemodialysis be carried out in diabetic patients who are not a part of an independent VA cooperative study.

MAJOR FINDINGS: New Contract.

CONTRACTOR: University of California, Los Angeles

AMOUNT: $ 93,000

TITLE: Nitrogen Metabolism in Uremia

OBJECTIVES: The objectives of this investigation are to study the metabolism of amino acids and their products and the enzymes which mediate the metabolism of these substances in uremia. It is believed that this information may help to define causes of uremic wasting and uremic toxicity, and may provide means of assessing optimal dietary treatment and dialysis regimens.

MAJOR FINDINGS: Histidine appears to be an essential amino acid not only in uremic patients but in normal man as well. Supplemental histidine does not appear to increase hemoglobin levels in uremic patients ingesting an adequate diet.

PROPOSED COURSE: They plan to continue their ongoing studies:

1. Continue to analyze amino acid content of plasma, muscle and liver from uremic patients.
2. Determine the dietary histidine requirements in normal and uremic subjects.
3. Determine the abnormal by-products of phenylalanine metabolism in uremia and perform toxicity studies.
4. Determine the locus of abnormal amine oxidase activity.

And, in addition, they will examine the role of Vitamin B_6 in the immunological responsiveness of uremic patients, and perform studies to establish the optimal amino acid intake in both acute and chronic renal failure.

Contractor: Cedar-Sinai Medical Center

Amount: $90,000

Title: Sorbent-based Regenerative Peritoneal Dialysis System

Objectives:

To develop a peritoneal dialysis system in which the dialysate is circulated through a sorbent cartridge for regeneration.

Major Findings:

The feasibility of the concept has been demonstrated using the same sorbent cartridge presently in use in the REDY® system. The contractor has developed a sterilization procedure for the cartridge using gamma radiation; and has built and tested a filtering system which practically eliminates particulate material from the fluid entering the peritoneal cavity. A prototype closed system peritoneal cycling machine has been constructed by CCI Life Systems Inc.

Proposed Course:

Longevity studies in uremic dogs will be conducted during the remainder of the present fiscal year. The principal effort in the next contract year will be to apply the technique to humans.

CONTRACTOR: Celanese Research Company

AMOUNT: $90,000

TITLE: Preparation and Evaluation of Optimized Hemodialysis Membrane

OBJECTIVES: To prepare superior membranes for hemodialysis from cellulose acetate and other polymers with emphasis being placed on practical results. The program is divided into three distinct phases (listed in order of priority): production (casting maching improvements, low hydraulic permeability CA membranes, membrane supply for clinical trials, intermediate hydraulic permeability membranes); development (optimization of membrane geometry/transport properties, PBI membranes, template membranes, hydrolysis of homologous cellulose esters); and some limited exploratory work.

MAJOR FINDINGS: Initially means were established to fabricate a state-of-the-art cellulose acetate (CA) dialysis membrane, culminating in the production of more than 800 square meters of such a membrane less than three months after the initiation of the contract. This membrane was characterized by high hydraulic permeability (6X Cuprophan PT-150) and superior metabolite permeability particularly to middle molecules (4.5 - 13X Cuprophan, based on Vitamin B-12 and inulin). Samples of the membrane were submitted for evaluation of pressure response, blood compatibility, toxicity, and transport characteristics and results were positive and corroborative of in-house findings. A clinical trial was recently initiated at the University of Washington and clinical experience to date has been excellent.

Laboratory studies centered initially on developing techniques for producing a CA membrane with lower hydraulic permeability (2-3X Cuprophan) to serve as a Cuprophan substitute. The methodology has now been developed and pilot production level efforts have been initiated. Subsequent development studies have led to a glycerinization technique that yields a "dry" membrane that can be handled under ambient conditions, a heparinization technique that is now being evaluated, and an in-depth study of viable casting solvents.

PROPOSED COURSE: Added emphasis will be placed on obtaining evaluation data from cooperating clinicians. The possibility of making a low cut-off experimental membrane will be considered.

Contractor: Children's Hospital, Boston

Amount: $50,627

Title: Trace Element Abnormalities in Children with Chronic Renal Failure

Objective:

The initial objective of the program was to define trace element abnormalities existing in children with chronic renal failure on hemodialysis or post transplantation, with particular emphasis on growth, development and nutrition.

Major Findings:

Preliminary data was obtained for plasma and red cell content of calcium, magnesium, iron, copper and zinc.

Proposed Course:

Termination.

Contractor: Cleveland Clinic Foundation

Amount: $50,000

Title: Development of New Concepts in Blood Access Devices

Objective:

Development of fistula shunts using thromboresistent biolized materials.

Major Findings:

The contractor has conducted animal experiments with a sheep model to confirm his previous findings with dogs. A shunt for human experimentation has been designed.

Proposed Course:

Human experiments will be conducted during the next contract year.

Contractor: University of Colorado

Amount: $77,820

Title: Trace Element Abnormalities in Chronic Uremia

Objective:

To determine the levels of concentration of trace elements in dialyzed uremic patients; to determine the clinical importance of trace element abnormalities, and to establish the concentration of trace elements required in the dialysate solution.

Major Findigs:

Considerable progress has been made in improving analytical techniques and tissue preparation for analysis. Plasma trace element constituents have been more fully characterized in control subjects. Preliminary studies have been carried out on dialysis patients in other geographical areas. Studies have been conducted to further characterize the body burden of a number of trace elements.

Proposed Course:

Studies will be continued using three methods of analysis. The proposed studies will attempt to determine:

(1) Tissue trace element profiles in chronic uremia
(2) Contaminants in dialysis equipment
(3) Transfer through membranes
(4) Geographical variations
(5) Trace element excretion in uremia
(6) Effect of duration of uremia on trace element levels
(7) Studies in some specific trace elements: Rubidium, Aluminum and Tin
(8) Special animal studies
(9) Epidemiological studies of the Dialysis Dyspraxia Syndrome

Contractor: Exxon Research and Engineering Company

Amount: $117,873

Title: Liquid Membrane Capsule System for the Treatment of Chronic Uremia

Objectives: To develop a liquid membrane to encapsulate reactants to be used as toxin traps in the treatment of chronic uremia.

Major Findings: The liquid membrane formulation has been demonstrated to be effective in removing ammonia from both solutions and synthetic gut fluid. Effective formulations have been used to encapsulate tartaric and citric acid. Animal experiments were continued.

Proposed Course:

The contractor will determine *in vivo* the efficacy of the capsules containing tartaric or citric acid on chronically uremic dogs. The experimental protocol includes evaluating the long term tolerance of capsules.

CONTRACTOR: Franklin Hospital

AMOUNT: $90,000

TITLE: Relationship of Uremic Abnormalities to Variable Molecular Weight Dialysis Therapy and Residual Renal Function

OBJECTIVES: The object of the studies is to investigate the mechanisms that control the concentrations of various solutes found in the chronic uremic and how they can be related to the patient's state of health. Proposed work concentrates, therefore, on the kinetics of nitrogen based protein catabolites (urea, creatinine, polypeptides) and the body water buffer system. The results of these studies are anticipated to provide new information about pathophysiologic mechanisms operative in dialysis and a data base for quantitative prescription and control of this treatment.

MAJOR FINDINGS: A need for accurate measurement of dialysis parameters, in order to document, and prescribe therapies quantitatively can be met by a clinical model. Information was gained on urea generation, distribution, and the kinetics. A similar evaluation was made with polypeptide material. Limitations of single pool modeling have made necessary a multicompartment model, which was used to evaluate the apparent volumes of distribution, the rate of generation and the intercompartmental transfer co-efficients of this material. Five uremic patients were treated sequentially at 6 month intervals with a cellulose hollow fiber kidney (CHFK), cellulose acetate hollow fiber kidney and CHFK. Observations on neuropathy, anemia and platelet function showed no change. The conclusion that these organ system lesions are unrelated to solute toxicity, however, cannot be justified since only a modest change in polypeptide concentrations would be predicted which could easily be overshadowed by dietary factors.

PROPOSED COURSE: Current plans are to continue this definitive investigation.

CONTRACTOR: Franklin Hospital

AMOUNT: $45,000

TITLE: Permeability of High Flux Dialysis Membranes in Operational Dialyzers with Variable Membrane Support Geometry

OBJECTIVES: The widespread use of kinetic modeling to guide experimental and therapeutic intermittent dialysis for uremia has resulted in a need for more precise knowledge of dialyzer solute transport rates. The overall goals of the proposed work are (1) further development of reliable and clinically applicable techniques to measure the in vivo volume rate of clearance relative to inlet plasma solute concentration in dialyzers with diverse geometry and membrane structure, (2) establish a firm data base for this parameter in these dialyzers and (3) develop reliable methods to calibrate membrane-dialyzer systems for in vitro and in vivo molecular sizing of endogenous solutes.

MAJOR FINDINGS: Methods have been developed and used in vivo and in vitro to measure ultrafiltration rate, whole blood clearance (K_b) whole blood clearance relative to inlet plasma concentration (K_d), plasma clearance (K_p), red cell clearance (K_{RC}), overall transport resistance (R_O) and and combined blood and dialysate resistance ($R_B + R_D$). These techniques have been used to measure these parameters with four high flux (HF) membranes and compare them to Cuprophan membrane (CM) in the D1 and D4 dialyzers for urea, creatinine, uric acid and SRI peptide in vivo and Raffinose, Vitamin B_{12} and Inulin in vitro. The D4 dialyzer was superior to D1 for all membranes. The HF membranes were all inferior to CM with respect to K_d for in vivo solutes but superior to Cuprophane for the in vitro solutes. The Q_F of HF membranes was 1.4 to 6 times Cuprophane in vitro but decreased in vivo to values ranging from 3 to 0.8 times Cuprophane. An overall leak rate of 54% was seen with high flux membranes compared to 0% with Cuprophane.

PROPOSED COURSE: Current plans are to discontinue this study at the end of the current period. Any additional data collection of this nature would be done as a minor part of the other Franklin Hospital contract.

Contractor: Gulf South Research Institute

Amount: $94,000

Title: Membranes and Materials Evaluation: Permeabilities, Physical and Mechanical Properties

Major Findings:

Detailed evaluation of three additional flat sheet membranes and two hollow fibers has been completed.

Proposed Course:

The evaluation of potential hemodialysis membranes, capillary fibers and materials in relation to their permeability, physical and mechanical properties will be continued. Additional solutes will be incorporated to evaluate membranes. Trace element content and transport through membranes, will be included in the forthcoming studies.

CONTRACT: Harvard University

AMOUNT: $58,000

TITLE: Multivariate Analysis of Dialysis Efficacy and Its Relation to Access Methods

OBJECTIVES: The study proposes to define the dialysis success profile (DSP) and measure it in a large group of patients. An attempt will be made to correlate dialysis success with measures of blood access success. The DSP is a quantitative three-part measure of dialysis efficacy. It is a composite of three factors; biochemical, disease-related, and psychosocial. Once defined and measured, it will permit group comparisons of patients by diagnostic category or by therapy schedule. DSP will be measured in a large stable group of chronic dialysis patients and the results correlated with patient, disease, and therapy-related factors. Particular attention will be given to correlation with details of access to the circulation. An hypothesis to be tested is that access efficacy, complications, and failures powerfully influence dialysis success.

MAJOR FINDINGS: The proposed dialysis success profile (DSP) has three components. The first is the biochemical and physiologic effect of dialysis. The second component of DSP is a record of symptoms and findings common to patients with end stage renal failure to provide a measure of the medical status of the patient. The third part of the DSP is an aggregation of psychological and social factors that express the result of the entire treatment experience in chronic hemodialysis. The contractor has partially completed (∿20%) the retrospective study of approximately 100 patients. For the second part of the DSP Medical Risk evaluation, ninety records have been reviewed and initial statistical analysis completed. For the third part of the DSP, the psychosocial variables, the contractor has completed data collection on fifty patients.

PROPOSED COURSE: When this retrospective study is completed at the end of the present contract period, the project will be completed.

CONTRACTOR: Indiana University

AMOUNT: $ 65,000

TITLE: Studies of Carbohydrate and Fat Metabolism in Uremia

OBJECTIVES: The goal is to elucidate the role of parathyroid hormone and glucagon in the abnormal carbohydrate and lipid metabolism in uremia.

MAJOR FINDINGS: Parathyroid extract in large doses acutely increases plasma glucose in the rat and in some normal human subjects. This action of the hormone on the liver is apparently mediated through cyclic AMP.

PROPOSED COURSE: Future studies will look at the role of a cyclic AMP mediated response to parathyroid hormone in the liver of human subjects. Additional studies will also be aimed at establishing the physiological role of glucagon, using the method of somatostatin suppression.

CONTRACTOR: Jefferson Medical College

AMOUNT: $ 83,858

TITLE: Amine Metabolism in Uremia

OBJECTIVE: To study the metabolism and toxicology of aliphatic amines in uremia with emphasis on correlating methylamine levels with uremic symptomatology.

MAJOR FINDINGS: The abnormal bacterial flora within the small bowel of uremic patients has been shown to generate trimethylamine. The elevations of duodenal dimethyl amine and increased numbers of bacteria occur when the serum creatinine approaches 8 mg%. Oral administration of nonabsorbable antibiotics decreases the serum methylamine levels. In one patient there was striking improvement in central nervous system symptoms which recurred when antibiotics were discontinued. The methylamines are volatile and appear on the breath accounting for in part the fishy amine odor.

PROPOSED COURSE: Dimethylamine and trimethyl amine levels will be correlated with uremic symptomatology. Patients will be dialyzed against a dialysis bath containing amine concentrations equivalent to their pre-dialysis serum levels thus allowing removal of other solute in exclusion to the methylamines. Clinical correlations will then be made.

CONTRACTOR: Jewish Hospital of St. Louis

AMOUNT: $ 55,792

TITLE: Bone Metabolism in the Chronic Uremic State and its Response to Vitamin D and Diphosphonatic Therapy

MAJOR FINDINGS: Four weeks of therapy with $25OHD_3$, Cl_2MDP or a combination of both in the chronically uremic rat has shown almost total reversion toward normal of the previously described defects in bone and collagen maturation. In addition the same regimens showed a dramatic reduction in soft tissue calcification.

PROPOSED COURSE: To elevate the effects of peritoneal dialysis on the skeletal maturational defect. To further analyze and identify the maturational defect of bone collagen. To evaluate the relative effectiveness of Vitamin D and Vitamin D analogues.

CONTRACTOR: Karolinska Institutet

AMOUNT: $51,570

TITLE: A study of Uremic Toxicology

OBJECTIVES: Utilizing the technique of high speed gel filtration various uremic body fluids have been separated into 10 components ultraviolet absorbing peaks. Distinct and readily recognizable differences in these peaks have been detected when uremic plasma and urine are compared to normal plasma and urine. The method of gradient elution appears to have an accuracy permitting quantitative analysis. Subsequent studies will be directed at attempting to correlate the absolute and relative magnitude of the sub-peaks of peak 7 to uremic symptomatology and clinical conditions. Quantitative analysis of these peaks will be used in evaluation of the influence of dialyzer designs, membranes, etc., on the removal of naturally occurring middle molecules. Attempts will also be made to further separate, isolate and identify the middle molecule peptides which accumulate in uremia.

MAJOR FINDINGS: The contractor has been able, utilizing the technique of high speed gel filtration to fractionate uremic body fluids into ten components ultraviolet absorbing peaks. With this technique more than 500 samples from 75 uremic patients and from normal subjects have been analyzed. One of these peaks (peak 7) in the middle molecular range is prominent in serum from patients with severe uremia but not detectable in normal serum. This peak was considered especially interesting in view of the middle molecular hypotheses for uremic toxicity. The occurrence of peak 7 also seem to correlate to the degree and severity of the uremic symptoms. However, this method proved to be at best semiquantitative thus only permitting screening between different categories of patients.

Peak 7 has been further separated into 7-9 different sub-peaks using the technique of gradient elution on DEAE Sephadex columns. Since the results can be quantitatively interpreted this technique offers a unique method for the evaluation of therapeutic modalities such as dialysis and dietary treatment. The magnitude of peak 7 and some of its sub-peaks seem to bear a relation to the severity of the uremic syndrome. Individual patients show characteristic peptide patterns but more extensive studies are needed to ascertain whether specific uremic symptoms can be related to an increase in a special peptide fraction or to a special pattern of relative intensities.

PROPOSED COURSE: This important and productive project will be continued.

CONTRACTOR: Mayo Foundation

AMOUNT: $ 80,000

TITLE: Uremic Neuropathy

OBJECTIVES: Studies are aimed at elucidating the underlying pathophysiology of uremic neuropathy, the long term course and possible prevention by early dialysis as well as the development of sensitive indicators of the development of this condition.

MAJOR FINDINGS: A touch-pressure instrument has been developed and appears to be a sensitive indicator of uremic neuropathy, No deterioration of peripheral nerve function has been found in patients dialyzed once a week while maintained on specific fluid and dietary restrictions.

PROPOSED COURSE:

1) To complete the creation of software, documentation of software and making of engineering drawings of the production models of touch-pressure and pallesthesia instruments so that software documentation and drawings may be made available to other dialysis centers and to manufacturers late in 1975 or early in 1976.

2) To obtain normative touch-pressure sensation and pallesthesia of finger and toe of man with age using the system III touch-pressure and pallesthesia instruments.

3) To test the relative precision of serial measurement of touch-pressure sensation, of pallesthesia and of nerve conduction to monitor changing nerve function before and after successful renal transplantation.

4) To study the effect of early versus conventional onset of hemodialysis on peripheral nerve function.

5) To compare short versus long duration of hemodialysis in patients dialyzed three times per week with special attention to peripheral nerve function.

CONTRACTOR: Minneapolis Medical Research Foundation

AMOUNT: $ 76,148

TITLE: Computer Data Bank to Assess Adequacy of Dialysis

OBJECTIVE: To compare dialysis conducted with dialysant flow rates of 500 ml/min and 200 ml/min.

MAJOR FINDINGS: Prolonged exposure (up to six months) of 10 patients to Q_D 200 dialysis has resulted in statistically significant reductions in serum cholesterol and elevations of MNCV. Concurrently, the patients arrived at a new steady-state of acid-base balance reflecting greater respiratory compensation of metabolic acidosis. These changes were assumed collectively to represent the effects of a 20% reduction in dialysis acetate load. Of particular interest is the duration of transients of small molecular weight substances (Urea, Cr, Uric Acid, and Phosphate). These substances appear to be still increasing even at six months into the study.

PROPOSED COURSE: The prolonged cross-over study of Q_D 500 vs Q_D 200 will be continued for an additional year.

Contractor: University of Minnesota

Amount: $80,000

Title: Fluid Dynamics of Blood Cells and Applications to Hemodialysis

Objectives:

The contractor has focused his research in four major areas: (1) Development of a novel blood access, (2) Development of a flowmetering unit for ultrafiltration rate, and (3) Deposition of formed elements onto filtering surfaces.

Major Findings:

A button type blood access device has been developed and tested; the ultrafiltration rate unit is at its final stage of development and extensive tests on deposition of platelets onto ultrafiltrating surfaces has resulted in correlations predicting the phenomena based on measurable parameters.

Proposed Course:

Work in all three areas will continue. Animal evaluation of the blood access device as well as the U. F. flowmeter will be conducted at the Minnesota Medical Research Foundation.

CONTRACTOR: University of Missouri

AMOUNT: $ 29,000

TITLE: Adherence of Blood Components to Artificial Kidney Membranes

OBJECTIVES: The purpose is to look at the question of antibody formation as a result of exposure to cellular debris in the course of hemodialysis.

MAJOR FINDINGS: Results are preliminary, but suggest that those patients who develop antinuclear antibodies while on dialysis may have poorer results with kidney transplants.

PROPOSED COURSE: Investigations will continue along the same lines, in order to provide longitudinal data as well as to yield a larger data base on which to base statistical conclusions. Patients on peritoneal dialysis will also be examined for the incidence of antinuclear antibodies.

CONTRACTOR: University of Naples

AMOUNT: $18,000

TITLE: Studies on Peritoneal Dialysis

OBJECTIVE: To continue a study which involves new approaches which promise to increase significantly the efficacy of peritoneal dialysis through the novel use of antidiuretic and vasodilating drugs in the peritoneal lavage solution, or of a combination of peritoneal lavage and the use of oral diuretics in the gut.

MAJOR FINDINGS: Dr. Giordano has demonstrated during the previous year of his NIH-supported research that inclusion of certain drugs in the peritoneal lavage solution significantly increases the transport of substances which must be removed out of the uremic patient through the peritoneal membrane. This development, in turn, promises to shorten significantly the time needed for peritoneal dialysis treatment for the maintenance of life in uremic patients.

PROPOSED COURSE: Selected patients, preferentially, patients with bilateral nephrectomy, will undergo short daily peritoneal dialysis. Small quantities of the following natriuretic drugs will be tried in the lavage solution: Furosemide 100 mg and Etacrinic acid 50 mg, as well as small quantities of the following vasodilators, Papaverin 30 mg, Nicotinic acid 50 mg, Beta pyridil carbinol 100 mg, and Isoproterenol 100 mg.

CONTRACTOR: University of Naples

AMOUNT: $28,000

TITLE: Studies on Oxystarch in the Treatment of Uremia

OBJECTIVE: To develop a chemical compound which can be administered *per os* to sequester urea and possibly other uremic toxicants in the gastrointestinal tract, and to develop with the aid of such a compound a suitable adjunctive treatment for patients in chronic renal failure. Also, to explore the feasibility of using oxystarch and related substances as a sorbent of uremic wastes to regenerate and recycle artificial kidney dialysate solutions.

MAJOR FINDINGS: Oxycellulose, a new polyaldehyde, has been obtained by slow oxidation of cellulose. Oxycellulose has a greater affinity than oxystarch to ammonia, and a somewhat reduced urea-binding activity. However, the major characteristic of oxycellulose is its insolubility, a property which makes it suitable for dialysis fluid regeneration. Another insoluble polyaldehyde was obtained by partial binding of oxystarch with ammonia. In vivo studies of oxycellulose were performed showing no detectable toxicity in mice who received 20% of their diet in the form of oxycellulose. In vivo studies in patients showed that when used as an intestinal urea sorbent, oxycellulose causes a significant decrease in the abnormally elevated BUN of these uremic patients and a corresponding increase in fecal nitrogen.

PROPOSED COURSE: In a long-term study oxystarch (with or without activated charcoal) will be given to patients (dialyzed and non-dialyzed) by mouth in the form of oxystarch-containing foods. The potential of this treatment for savings in dialysis time will be studied for one year. If successful, a possible application to patients equipped with indwelling peritoneal catheters is envisioned; the oxycellulose or oxystarch with charcoal will be utilized to regenerate the peritoneal dialysate.

CONTRACTOR: National Institute of Scientific Research

AMOUNT: $160,000

TITLE: Modified Polycarbonate Membranes for Hemodialysis

OBJECTIVES: The objective is to prepare an improved hemodialysis membrane of lower transport resistance and higher mechanical strength than Cuprophan®. Scope of the work involves solving a number or processing problems such as: 1) scale-up of polymer synthesis; 2) control of polymer molecular weight; 3) evaluation of other solvent systems for casting; 4) development of improved quality control; 5) elimination of pinholes; 6) development of a process for dry-storable membrane; 7) definition of a suitable sterilization procedure. In addition initial clinical evaluation will be done by subcontract.

MAJOR FINDINGS: Earlier in the contract two series of copolyetherpolycarbonates derived from Bisphenol A and polyether glycols of varying molecular weight were synthesized and characterized. Trends in hydrophilic character and mechanical strength of the copolymers were established and a single copolymer was selected for dialysis membrane development. Gelled membranes prepared from this copolymer exhibited good transport properties for dialysis of various solutes. The membranes were superior to Cuprophan® in their permeabilities to solutes in the "middle molecule" range (300-5,000 molecular weight) and their ultrafiltration rates were 2 to 5 times that of Cuprophan® with no albumin leakage. Blood compatibility tests showed their thrombogenicity to be comparable to that of Cuprophan®, and acute toxicity screening tests revealed no toxic effects. Other accomplishments have included: scaling up the synthesis of polymer to one-pound batch size; a process for water gelation (rather than a more expensive and hazardous solvent gelation) was developed; a continuous casting process (up to several hundred feet lengths) for membrane 24 inches wide was demonstrated.

PROPOSED COURSE: The project will be completed at the end of the current contract.

CONTRACTOR: University of North Carolina

AMOUNT: $ 70,000

TITLE: Antithrombogenic Surfaces: Platelet-Interface Reactions

OBJECTIVE: To obtain a better understanding of the interaction between blood and artificial surfaces.

MAJOR FINDINGS: Work has proceeded in six different areas. 1) An in vitro system for exposure of native blood to dialysis membranes in the absence of other artificial surfaces has been devised and evaluated. 2) A study has been made of the plasma proteins which adsorb to Cuprophane and other artificial surfaces. Tentative identification has been made of certain of these adherent protein species. 3) A study has been made of the adhesion of platelets to Cuprophane and other artifical surfaces. Platelets in normal plasma were less adherent to Cuprophane than to polyethylene or polystyrene and adhesion of Cuprophane was further reduced in uremia. 4) Certain artificial surfaces were shown to activate Factor XI on the platelet plasma membrane. Artificial surfaces were shown to initiate blood coagulation through at least four different routes. 5) Test devices exposed to flowing blood in arteriouvenous shunts in rhesus monkeys were used to evaluate the antithrombotic effects of a number of pharmacologic agents. Cyproheptadine was shown to be the most effective antithrombotic agent under these test conditions. 6) Human endothelial cells were shown to grow in tissue culture upon Cuprophane, polyethylene, and polystyrene. Endothelial cells were markedly adherent to polyethylene and polystyrene but less adherent to Cuprophane.

PROPOSED COURSE: This contract terminates in June 1975. The principal investigator will continue his investigative efforts with a new contract from NIAMDD at The Memorial Hospital, Pawtucket, Rhode Island. Future work will be in 4 areas:

1) Studies on inhibition of adhesion of platelets to Cuprophane and other artificial surfaces. This will include work on characterization and partial purification of a naturally occuring inhibitor of platelet adhesion to artificial surfaces which has been discovered in plasma.

2) Radioimmunologic studies of plasma proteins adsorbed to artificial surfaces.

3) Studies of human endothelial cells grown in tissue culture upon artificial supporting media such as that used in artificial kidneys.

4) On-Call Service to AK-CUP including:

   a) in vitro screening of the compatibility of artificial surfaces with blood.

   b) studies, as needed, on the interactions of materials prepared for use as sorbents in blood with various components of blood.

CONTRACTOR: Northwest Kidney Center

AMOUNT: $ 42,000

TITLE: A Comparison of Home Hemodialysis and Home Peritoneal Dialysis in Patients Trained at a Community Dialysis Center

OBJECTIVES: This study will compare the overall medical effectiveness of home treatment by peritoneal dialysis with that of home hemodialysis in similar patients.

MAJOR FINDINGS: The back-up dialysis rate for medical reasons in peritoneal dialysis patients is no greater than for hemodialysis patients. The overall mortality for patients on peritoneal dialysis has been approximately half as great again as for patients on hemodialysis; this undoubtedly relates to the patients selected for peritoneal dialysis.

PROPOSED COURSE: The study will compare medical effectiveness, incidence of complications, degree of social and vocational rehabilitation, and cost of the 2 forms of treatment. In addition, special studies will be performed to compare the effects of peritoneal dialysis and hemodialysis on platelet survival and lipid metabolism.

CONTRACTOR: University of Pisa

AMOUNT: $25,095

TITLE: Albumin and Hormone Metabolism in Chronic Uremia and Hemodialysis

OBJECTIVE: To elucidate the turnover and biochemical aberrations of albumin, insulin, and growth hormone metabolism in uremia both on and off dialysis.

MAJOR FINDINGS: Sophisticated determination of albumin catabolism through a new triple tracer technique has shown that the intravascular albumin mass is normal in patients on the low, selected protein (Giordano-Giovannetti) diet (even in patients maintained on it up to five years). The extracellular albumin mass, total albumin mass and extracellular to intravascular ratio is reduced in both short-term and long-term patients. The total intravascular albumin mass is preserved within a normal or near normal range. With regard to insulin it was found that catabolism of insulin was slowed down considerably in uremic patients -- apparently because normally functioning renal tissue is required for a normal rapid breakdown of insulin in the body. It was also found, however, that the rate of secretion of insulin from the pancreas is reduced in uremia -- apparently because of an as yet unknown toxic suppression of normal insulin synthesis in the pancreas or secretion from the beta cells.

Chronic dialysis of several months' duration distinctly improved the fractional catabolic rate, metabolic clearance and insulin delivery rate which are markedly reduced in uremic patients before dialysis treatment. After dialysis of several months these values approach normal values in a near-asymptotic fashion.

PROPOSED COURSE: Studies of ^{125}I-insulin kinetics in uremic patients submitted to regular dialysis program will be continued; these studies are performed at various intervals from the preceding dialysis. Aim of these studies is to evaluate the relationship between insulin metabolism and the plasma concentration of toxic metabolites.

Similar studies are proposed for growth hormone using basically the same techniques and a new method developed by the group which has already been used successfully in HGH kinetics feasibility studies. They wish to investigate whether prior to and immediately after dialysis radioactive-labelled growth hormone is present in different amounts. They also intend to study growth hormone status on a long-term basis in patients on dialysis. Since the observed high human growth hormone levels may possibly be a factor in the hypertriglyceridemia of uremia, the proposed study of human growth hormone levels in relation to the frequency of dialysis may yield information of value concerning enhanced atherosclerosis in uremic patients on dialysis.

CONTRACTOR: Research Triangle Institute

AMOUNT: $169,950

TITLE: National Dialysis Registry

PROJECT DESCRIPTION: The contractor will continue to accumulate data for the National Dialysis Registry on chronic intermittent dialysis patients, and quarterly statistical printouts of the total registry patients to the Project Officer and cooperating centers. They will furnish the same data on patients in VA centers to the Project Officer

Data files which link patient or center names with data will be maintained in a manner which insures the maintenance of confidentiality of patient and center data; the manner in which these files are handled have been reviewed and approved.

The National Dialysis Registry has collected data since 1969 from virtually all centers engaged in dialysis in the United States, and prepared from the data statistical analyses for use by the NIAMDD and quarterly reports to all cooperating centers. The data include numbers of patients in center or home dialysis, changes in status of these patients, cause of death in patients that expire, length of time on dialysis, etc. Confidentiality of the center and patient identities is maintained. Analyses of death by primary cause and multiple decrement life tables are also now available.

PROPOSED COURSE: Current plans are to phase out this contract at the end of its present term after transferring any necessary data to the new ESRD Medical Data System to be operated by BQA.

CONTRACTOR: University of Rochester School of Medicine

AMOUNT: $ 80,000

TITLE: Prospective Study of Cytomegalovirus and Herpes Simplex Virus Infection of Patients Undergoing Chronic Hemodialysis

OBJECTIVE: In an effort to understand the etiology of fever, pneumonia, persistent pericarditis and gastrointestinal problems in hemodialysis patients, they have undertaken a prospective study of cytomegalo virus (CMV) and herpes simplex virus (HSV) infection and antibody response to these viruses.

MAJOR FINDINGS: This report confirms our initial observations that cytomegalovirus (CMV) shedding occurs in 17% of antibody positive hemodialysis patients and is a reactivation of latent infection. Acquisition of CMV antibody is at most a rare event by dialysis patients and personnel but occurs frequently in transplant recipients. Evidence is presented that latent virus transplanted in the kidney explains this acquisition of CMV antibody and the production of new antibody precipitates a primary infection syndrome and a high incidence of renal malfunction and nephrectomy. Reactivation of CMV infection occurs in virtually all pretransplant antibody positive patients and although it was not associated with a primary infection syndrome it was associated with renal malfunction and nephrectomy. Thus CMV infection was associated with 30% return to dialysis whereas only 6% of uninfected patients returned to dialysis. Studies with influenza vaccine confirmed that dialysis patients respond intermediate between renal failure patients and normals with part of the defect being amount of antibody produced. Response in transplant patients to influenza vaccine correlates with creatinine clearance, being much less than 70 ml/min.

PROPOSED COURSE: Using the already collected serum specimens as well as the serum specimens that will be collected in our continuing prospective study, we will measure different types of antibody activity and correlate this data with data of virus shedding collected simultaneously. Emphasis will be placed on measurement of IgM, complement enhanced neutralizing, and cytolytic antibody activities that our initial studies have shown to be closely associated with the development of renal malfunction. Our studies will also include evaluation of the serologic status of the donor in an attempt to define what the effect of transplantation of a virus containing kidney to either a virus negative or a virus positive recipient.

CONTRACTOR: University of Southern California

AMOUNT: $ 75,000

TITLE: Osteodystrophy and Divalent Ions in Kidney Failure

OBJECTIVE: To carry out studies to evaluate various aspects of deranged divalent ion metabolism in renal failure. To evaluate the effectiveness of $1,25(OH)_2D_3$ and $1\text{-}\alpha\text{-}OHD_3$ in correcting the abnormalities of divalent ion metabolism.

MAJOR FINDINGS: To date studies have demonstrated that patients with early, moderate, and chronic renal failure as those with acute renal failure display skeletal resistance to the calcemic action of parathyroid hormone. Intestinal absorption of calcium is defective in patients with renal failure and this defect is probably located in the proximal small bowel and is corrected by $1,25(OH)_2D_3$.

PROPOSED COURSE: During the forthcoming year the investigators will evaluate the effect of phosphate restriction on secondary hyperparathyroidism and the apparent skeletal resistance to parathyroid hormone, evaluate the use of high oral calcium intake in overcoming the impaired intestinal absorption of calcium and further evaluate $1,25(OH)_2D_3$ and $1\text{-}\alpha\text{-}OHD_3$.

Contractor: Southern Research Institute

Amount: $76,000

Title: Activated Carbon Fibers for Use in Artificial Kidney Devices

Objectives:

To develop activated carbon fibers suitable for use in artificial kidney absorption devices, and to develop activated carbon hemoperfusion cartridges.

Major Findings:

Fibers with carbon loading of 80% have been prepared in wetspinning facilities and wound into coils. Cartridges containing these coils have been evaluated to determine their capacity to remove uric acid and creatinine from aqueous solutions.

Proposed Course:

Two hundred devices will be fabricated and sterilized by autoclaving for evaluation. The evaluation program includes determination of:

(1) Cleanliness, sterility, mechanical strength and storage stability.

(2) Acute and subacute toxicity of the cartridge
(3) Effect of the device on hematology and blood chemistry *in vivo*
(4) Ability of the cartridge to remove a variety of compounds from the blood.

CONTRACTOR: Stanford Research Institute

AMOUNT: $ 60,000

TITLE: Artificial Kidney - Biochemical Evaluations

OBJECTIVE:

1. Determine the dialysance of amino acids and their non-protein conjugates, including peptides, during hemodialysis of patients employing new artificial kidney systems.

2. Examine dialysates obtained during hemodialysis with various artificial kidney systems for the presence of proteins of low molecular weight and peptides (1,000 to 10,000 daltons), and relate this information to similar materials in uremic plasma.

3. Attempt to relate removal or retention of small proteins or peptides in this molecular weight range to the clinical response of uremic patients to various modes of hemodialysis.

BASIC FINDINGS: Plasma from uremic patients and normal subjects was grossly fractionated into proteins, free and conjugated amino acids (Peak II), and uric acid. Fractionation was assessed by flow-monitoring at 280 nm, immunoelectrophoresis, fluorescamine and phosphotungstate reactivity, and measured content of amino acids before and after hydrolysis of collected fractions. Conjugated amino acids of Peak II were very low in normal subjects compared with uremic patients. The 280-nm absorption was significantly correlated with the total content of conjugated -amino N_2, but not with that of the unconjugated -amino N_2. Generation rates of the Peak II materials were directly related to the generation rates of urea, but not with those of creatinine. Thus, Peak II materials were derived from catabolism of ingested protein as is urea. Clearances of the Peak II materials by experimental high-flux membranes were not found to exceed those measured for cuprophane. Conjugated amino acids were separated from amino acids of Peak II by ligand and exchange chromatography and were further fractionated on Sephadex G-15. Hippuroc acid, phenylacetylglutamine, and an acidic peptide containing principally aspartic and glutamic acids and glycine were identified.

PROPOSED COURSE: Isolate and characterize "middle molecular weight: peptides from biological fluids of uremic patients.

Develop procedures for the rapid estimation of these compounds that can be applied by well equipped clinical laboratories.

Examine possible correlations between the levels of these compounds and the pathophydiologic characteristics of uremia.

CONTRACTOR: Stanford University

AMOUNT: $ 75,000

TITLE: Hyperglycemia, Hyperlipidemia, and Insulin Resistance in Chronic Renal Failure and in Relation to Hemodialysis

OBJECTIVES: The goal of these studies is to provide an understanding of the abnormal carbohydrate and lipid metabolism that occurs in patients with chronic renal failure.

MAJOR FINDINGS: Triglyceride levels are increased but not significantly in fasted acutely uremic rats. The slight increase in triglyceride levels in the presence of a marked decrease in triglyceride secretion suggests that decreased triglyceride removal is the major defect in triglyceride metabolism in acutely uremic rats.

PROPOSED COURSE: Future studies will examine uremic hypertriglyceridemia and glucose intolerance in man, the effect of hemodialysis on glucose intolerance in uremia, and studies of insulin resistance in animal models.

Contractor: University of Tennessee

Amount: $35,000

Title: Detection of Toxicity of Extracted Constituents from Dialyzers and Components

Objective:

To develop *in vitro* tests to determine toxicity of dialyzers and components.

Major Findings:

The contractor has developed four tests which are sufficiently sensitive for the proposed goal.

Proposed Course:

The contractor will test ten commercially available dialysis units to obtain a base line on toxicity based on his four *in vitro* tests.

CONTRACTOR: Vanderbilt University School of Medicine

AMOUNT: $ 100,000

TITLE: Neurobehavioral and Clinical Response to Hemodialysis

OBJECTIVES: The aim is to develop objective, quantitative measures of uremic dysfunction of the nervous system in patients with renal failure 1) as indicators of adequacy of dialysis and 2) as tools for investigating the pathogenesis of uremic neurobehavioural impairments.

The investigators have developed several tests at various levels of nervous system function that correlate with the degree of renal failure and improve with dialysis.

MAJOR FINDINGS: In sixty carefully managed patients with end-stage renal disease several quantitative measures of peripheral nerve function, slow-wave-associated EEG power, visual stimulus processing, and cognition dependent performance were obtained before and after onset of maintenance dialysis or transplantation. The results reveal (1) significant direct correlation of cognitive impairment, slow-wave EEG power, visual stimulus response delay, and certain photic driving responses (but not of peripheral nerve conduction velocity, distal latency or potential amplitude) with the serum creatinine concentration as a measure of renal failure, (2) return of these objective measures toward normal along with clinicians' and patients' subjective estimates of improvement after onset of maintenance dialysis, and (3) measured improvement that varies directly with time and frequency of dialysis, renal transplantation, and reversal of transplant rejection.

PROPOSED COURSE: They propose to use neurobehavioral response parameters to determine the effects of, and to optimize the relationships between, quantitatively measured and controlled metabolite generation and various dialysis formats, in patients' with chronic renal failure, in order (1) to optimize dialysis treatment, (2) to provide an objective basis for estimating dialysis cost/patient benefit relationships, and (3) to explore further the pathogenesis of clinically significant dialysis-responsive uremic impairment.

CONTRACTOR: University of Washington

AMOUNT; $ 70,000

TITLE: Acid-Base Chemistry and Human Bone

OBJECTIVE: To understand better the various features contributing to uremic osteodystrophy and to their various interrelationships, and thereby to design treatment modalities which will delay, improve or prevent the skeletal disabilities encountered in patients with chronic renal failure.

MAJOR FINDINGS: The investigator has identified in human iliac crest biopsy material obtained from patients treated by hemodialysis abnormalities of magnesium, carbonate, true density and Ca/P which correlate with quantitative histology and which are progressive in severity from mild, malacic to fibrotic histology. In an attempt to assess various treatment modalities via double biopsies (one year apart) measurement of biochemical and histologic parameters reveal that a 3.0 mEq [Ca] dialysate results in no improvement in osteodystrophy, a 4.0 mEq [Ca] results in improvement ot bone CO_3; and supplementation with oral $CaCO_3$ results in improvement of bone CO_3 and Mg with the latter program being the only one resulting in biochemical parameters nearing normal.

Using a rat model the investigator has demonstrated that the plasma Ca/Mg ratio determines bone Mg and that this is then associated with decreased bone CO_3 and Ca/P ratio. Uremia and/or acidosis results in a decreased bone density.

PROPOSED COURSE: The investigator will continue biochemical and histologic analyses of current biopsy material. Approximately 12 patients with advanced renal osteodystrophy will be dialyzed against a magnesium free dialysate. Rats will be used to further evaluate the effects of PTH and Ca/P on the biochemical and histological bone lesion associated with uremia.

CONTRACTOR: University of Washington

AMOUNT: $ 58,000

TITLE: Study of Hyperlipidemia and Atherogenesis in Renal Failure

OBJECTIVE: To discuss the mechanisms of hyperlipidemia in chronic renal failure and the post-transplantation state.

MAJOR FINDINGS: Lipoprotein concentration and composition have been determined in undialyzed, dialyzed and transplanted renal patients. Uremic patients demonstrated increased triglycerides and normal cholesterol in whole plasma, and increased triglyceride content in both VLDL and LDL. Transplant patients had increased triglyceride and cholesterol in whole plasma.

Using a chronically uremic rat model lipoprotein lipase was quantitated in the heart, diaphragm and adipose tissue. Tissue activity in the fed and fasted states did not differ in control and uremic animals. Corticosteroid immunosuppression (uremic rat) was found to produce hypertriglyceridemia and a significant reduction in adipose tissue lipoprotein lipase, suggesting that impaired removal may contribute to triglyceride elevation in renal transplant patients.

PROPOSED COURSE: The investigators will further characterize the impairment in lipoprotein catabolism which seems to be present in chronic uremia and to explore the status of the triglyceride-removal mechanisms in transplant patients.

CONTRACTOR: University of Washington

AMOUNT: $ 135,000

TITLE: Clinical and Engineering Research on Hemodialysis Procedures

OBJECTIVES: The aim is to determine what constitutes minimum dialysis and optimum dialysis.

MAJOR FINDINGS: They have been able to maintain patients on markedly decreased levels of dialysis, and have tried to quantitate the amount of dialysis for each patient in terms of a dialysis index. Nerve conduction velocity would appear to be the most sensitive index for insufficient dialysis.

PROPOSED COURSE: They will continue the refinement of the mathematical model for a dialysis index, and expand the number of patients in the reduced time protocol.

CONTRACTOR: University of Washington

AMOUNT: $ 80,000

TITLE: Cannula Research

OBJECTIVE: The objective is the improvement and development of hemodialysis cannulae.

MAJOR FINDINGS: In prior work the concept of the fistula catheter has been formulated and subjected to testing in animals. Single fistula catheters implanted in fistula veins in the neck or leg of sheep remained functional and thrombus-free up to six months. The testing of additional catheter design prototypes demonstrated the importance of utilizing extravascular catheter reinforcement and the lack of need for reinforcement of the intravascular portion of the catheter. Catheters were shown in pumped in vivo studies to have acceptable pressure drop at dialysis blood flows when used as single access systems and also as dual access (two catheter) systems. Catheter collapse did not occur at dialysis blood flows during these studies. In recent animal work, we have demonstrated that significant improvement in thrombus-free function is achieved when catheters are inserted in the proximal fistula vein instead of at the fistula.

Also, in recent work several dialysis patient volunteers have received single fistula catheters. Pumped blood flow rates during thrice weekly dialysis have ranged from 200-40 ml/min. Implants remain functional up to 13 months.

PROPOSED COURSE: As a continuation of prior investigative effort, a vascular access device, the fistula catheter will be implanted in selected dialysis patient volunteers. Catheters will be used for dialysis treatment and catheter performance assessed for blood flow rates achieved, and for complications associated with catheter implantation and usage in order to characterize the overall applicability of the implant to patient blood access needs. In related work, up to 40 sheep will receive experimental immobilized fistula catheters in fistula veins. Performance of catheters with no orifice (controls), and with one or more orifices will be characterized for thrombosis and for suitability for pumped blood withdrawal and return. Results will be compared against data for unimmobilized catheters which were studied in prior animal experiments.

CONTRACTOR: University of Washington

AMOUNT: $59,690

TITLE: Anticoagulant-Bound Hemodialysis

OBJECTIVES: The objective during this second contract year is to further refine and test methods of heparin grafting of the entire hollow fiber hemodialyzer system including blood lines, drip chambers and hollow fiber dialyzer. Continual testing of the experimental grafted system is planned using sheep. In addition to routine coagulation studies, kinetic studies of Cr^{51}-tagged platelets and I^{125}-labeled fibrinogen will be performed with heparin grafted and non-grafted (control) dialyzers and tubing sets. Studies to assess the degree of change in dialyzer membrane permeability resulting from variations in heparin grafting technique will be undertaken by in vitro and in vivo clearance determinations of creatinine, urea and B_{12}. Clinical testing of grafted dialyzer and tubing assemblies which exhibit acceptable antithrombogenic and permeability characteristics, as determined by animal studies, is anticipated during the second half of the contract year.

MAJOR FINDINGS: Using the cyanogen bromide method, heparin has been bound covalently to the Hollow Fiber Artificial Kidney (HFAK) Cordis-Dow Model III, retaining biological activity as an anticoagulant. Three different reaction conditions for the binding of heparin to the hollow fibers (HF) have been applied and subjected to in vivo test. In vivo testing of these three series in sheep using a carotid-jugular double shunt system revealed increased antithrombogenicity of the heparin-bound HFAK. While blood flowrates in heparin-grafted HFAKs series A, B and C remained fairly stable at about 200 ml/min throughout the experiments (7 hours), a progressive decrease of blood flowrate to less than 100 ml/min within 5 hours was found in non-grafted dialyzers. In vitro clearance in heparin-grafted kidneys showed a decrease of creatinine clearance after grafting from 85 ml/min to 77 ml (minus 10%) but an appreciable decrease of vitamin B_{12} clearance from 12.0 ml to 7.0 ml/min. This reduction in membrane permeability has to be minimized to use the heparin grafted HFAK in the treatment of chronic uremia. Heparin entrapment into poly vinyl pyrrolidone (PVP) hydrogen layer grafted to poly vinyl chloride (PVC) blood tubing sets was achieved by radiation grafting with Cobalt 60.

PROPOSED COURSE: In the latter part of the present contract year, or perhaps in the next depending on data adequate to justify human experimentation, clinical trials will be started. When adequate clinical data has been accumulated to characterize the potential clinical benefits this study will be completed.

ANNUAL REPORT SUMMARY
EPIDEMIOLOGY AND FIELD STUDIES BRANCH

Southwestern Field Studies Section

The Section has continued to focus primarily on the epidemiology of diabetes mellitus, and in particular, on its distribution in the Pima Indians. The initial group of Pima Indians studied by the Section has now been under surveillance for diabetes for a ten year period. As a consequence of the length of follow-up it is now possible to evaluate factors which precipitate diabetes and which accelerate, precipitate, or ameliorate the vascular complications of diabetes mellitus.

During the past year factors relating to the appearance of glucose intolerance have been investigated. Serum insulin levels among subjects who subsequently developed unequivocal diabetes were found not to differ from those among subjects who remain normal when the degree of obesity at baseline was considered. The insulin responses to oral glucose loads did not differ at baseline in groups whose glucose tolerance deteriorated and those who did not. It was also found that the insulin response did not change appreciably with the advent of glucose intolerance suggesting that insulin response *per se* does not play an important part in the process of decompensation. Analysis of sequential glucose tolerance tests, however, showed that Pima Indians who had modest elevations of the post-load glucose level and who were more obese developed diabetes much more frequently than age-matched control subjects.

As a consequence of these findings the development of mild degrees of glucose intolerance and the patterns of growth and development as they relate to the development of obesity have been examined in Pima children from age five and upwards. The differential patterns of growth and development in Pima Indian children as compared to Caucasian children have been described. Differences were found to result from a more rapid weight gain in the seven to eleven age range and beyond age eighteen years. This second phase of weight gain corresponds well to the development of mild degrees of carbohydrate intolerance in many individuals.

The pattern of development of serum cholesterol levels has also been examined between birth and adult years. This was felt to be important because of the associations between serum cholesterol and coronary heart disease seen in Caucasians and in view of the fact that Pima Indian cholesterol levels remain constant from age 25 and onwards. While Pima Indian cholesterol levels were found to be similar to Caucasians at birth and up to five years of age they were subsequently diverged from those of Caucasians. The phases of increase occurred at similar ages in childhood and adolescence, but the rise ceased between age 20 and 24 in contrast to Caucasians, who continue to rise into the fifth and sixth decades. Among Pima Indian females it was also found that there was no elevation of cholesterol level associated with the occurrence on menopause.

The purported association between diabetes and tuberculosis has been confirmed and the magnitude of the association has been documented.

The availability of ten year follow-up data on a substantial number of Pima Indians provides the most extensive information ever gathered on the natural history and development of diabetes and on its vascular complications. These data will be examined in many ways to determine whether factors which are responsible for the initiation, progression, or delay of any of these events may be identified.

Since diabetes is believed to be inherited, the period between birth and the appearance of clearly identifiable manifestations represents a time in which the basic biochemical or morphological characteristics of the basic defect of diabetes may be recognized. Since many prediabetic subjects have been identified amongst the Pima Indian population a careful characterization of these individuals has been performed.

To the present time only insulin and glucagon analyses have been completed. Following a rapid intravenous glucose tolerance test it has been shown that the prediabetic (and the normal) Pima Indians have insulin levels which are two to three fold higher than those of Caucasians. This difference cannot be explained on the basis of differences in weight or other yet identified variables. The differences in insulin levels need considerable exploration and investigations are now underway to ascertain the biological effectiveness of the circulating insulin and investigation of insulin receptors in Pima Indians will be undertaken.

Glucagon levels, on the other hand, are similar in prediabetic and normal Indians and Caucasians, indicating that an abnormality in glucagon secretion is unlikely to be a genetic marker for diabetes. Glucagon levels were, however, shown to be elevated in recent onset Pima Indian diabetics. This elevation occurred regardless of the serum insulin level, and was found to be just as severe in diabetics with milder degrees of glucose intolerance as in those with more severe degrees of glucose intolerance.

A further line of investigation to quantify the relative importance of insulin and glucagon in the genesis of diabetes in the Pima Indian will be undertaken by examining the pancreas of Pima Indians autopsied by quantitative microscopy.

Metabolic Disease Epidemiology Unit

This Unit has continued to characterize the antithyroid compound derived from E. coli. This has been characterized as a true goitrogen as it blocks the organification of radioiodine in the rat thyroid. Partially purified material has been shown to be immunologically active, and a test for the presence of antibody to the goitrogen using a hemagglutination technique has been devised. Chemical characterization of the material indicates that it is a conjugated protein either glycoprotein, lipoprotein, or nucleoprotein.

Project No. Z01 AM 69,000-10 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Prospective Study of the Natural History of Diabetes Mellitus in the Gila River Indian Community

Previous Serial Number: NIAMDD-EFS-2(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Peter J. Savage
Dr. Richard F. Hamman
Dr. Stephen L. Aronoff
Dr. Max Miller
Dr. Elmer J. Ballintine
Dr. Phillip Gorden
Dr. Jesse Roth

Cooperating Units: Indian Health Service
Case Western Reserve University
National Eye Institute
Diabetes Branch, NIAMDD

Man Years:

Total:	6.9
Professional:	1.6
Others:	5.3

Project Description:

Objectives:

1. Determine the prevalence and incidence of diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from prediabetic state to subclinical diabetes mellitus and to clinical diabetes.

3. Study the association between diabetes and rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods Employed:

1. The following tests and examinations are repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River reservation.

   a. Clinical interview
   b. Physical examination
   c. Modified glucose tolerance test

and in addition on those aged 15 years and over

   d. Fundus photographs
   e. Electrocardiogram
   f. Tests for vibratory sensation threshold
   g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations will also be done on Indians of other tribes or living under different environmental conditions for comparison.

Major Findings:

1. A study of insulin levels over a wide spectrum of glucose tolerance has been completed and the findings are to be published.

2. Insulin levels as predictors of subsequent glucose tolerance: No significant difference was found between insulin levels of subjects with normal glucose tolerance who remained normal and those who subsequently developed diabetes when subjects were matched for 2 hour glucose level and degree of obesity on initial test. Additional studies utilizing multiple regression analysis to determine the difference between predicted and observed insulin values have extended these findings on a larger number of subjects. When corrected for glucose level and degree of obesity insulin levels have no value in predicting subsequent glucose tolerance 2 to 6 years later.

3. Sequential insulin measurements: Insulin responses on two oral glucose tolerance tests performed on average 2.5 years apart were examined in 78 subjects to determine if any particular patterns were associated with changing glucose tolerance. Insulin responses of groups were relatively stable despite significant changes in glucose levels. The data suggest that factors, other than changing insulin levels, are important in the development of glucose intolerance.

4. Cholesterol levels in the Pima Indians: The Pima Indians have previously been reported to have lower levels of serum cholesterol than their Caucasian counterparts. Cholesterol levels in children were evaluated. A sample of cord blood from 29 newborns showed a level of 86±3 mg/100ml for males and 88±2 mg/100ml for females. Between birth and five years of age the level of the serum cholesterol increased.

From five years of age to fourteen years of age, the level of cholesterol remained stable with a mean level of 145±1 mg/100ml for males and 144±1 mg/100ml for females. A rise in cholesterol was observed at about 17 years of age and continued through the early 20's. The mean serum cholesterol level from 15 to 19 years was 152 mg/100ml for males and 158 mg/100ml for females.

5. Cholesterol levels at menopause were determined on a large group of Pima Indian females. Out of 65 women who could be clearly identified as having cholesterol level measured just prior to and after menopause there was no indication of elevation of cholesterol levels between the two points. No evidence was found to support the hypothesis that the menopause is associated with an increase in serum cholesterol level. Diabetic women were found to have significantly higher serum cholesterol levels than non-diabetic women in the 34-54 age group. (186±3 mg/100ml vs. 176±3 mg/100ml). These findings have been reported.

6. Growth and development among Pima Indian children: The Pima Indians are more obese than non-Indian populations. The adult Pima Indian has been shown to weigh on average 150 percent of desirable body weight. To establish the pattern of development of obesity, Pima Indian children were examined between the ages of 5-25. The mean weight of a Pima child at age 5 was 21.0 kilograms for males and 20.4 kilograms for females, which is similar to Caucasian norms. Between the ages of 7-11, there is a gradual and much more rapid weight gain than that found among Caucasian children. By age 11, the average Pima male weighed 47.1 kilograms and the average female 48.4 kilograms, which is 28 percent above the respective Caucasian means. This very striking increase in weight during pre-pubertal years was not associated with an increase in the mean two-hour plasma glucose.

   Weight gain from 12 to 18 years in Pimas was similar to that of Caucasians. From age 18 to 25 the weight of the Pima once again increased. At age 25 males weighed 91±3 kilograms, females weighed 82±2 kilograms. These weights are 28 percent and 37 percent above mean weight for Caucasian adults. During this period mean two-hour plasma glucose increased from 103 to 140 mg/100ml.

7. Analysis of sequential glucose tolerance tests: Bimodality of two-hour plasma glucose levels in the Pima has been described. Two-hour plasma glucose levels in 762 subjects aged 25-54 who had had at least two glucose tolerance tests were analyzed to describe evaluation of glucose tolerance. Plots of fasting vs. last two-hour glucose gave four clusters of subjects who could be identified as 1) stable normals, 2) stable diabetics, 3) new diabetics, and 4) remitters. Analysis of these groups indicated that new diabetics had significantly higher initial two-hour plasma glucose and percent desirable weight than the stable normals. The remitters had significant weight loss between first and last exams. Life table analysis of the same data confirmed that both high initial two-hour glucose level and high initial percent

desirable weight conferred significantly higher risk of decompensation. When both were present they acted synergistically to produce 15 times the risk of decompensation over an 8 year period as compared to subjects with normal weight and a low two-hour plasma glucose.

8. Association between diabetes and tuberculosis: Both diabetes and tuberculosis are major disease problems of American Indians and an association between the two has been postulated. The observed and predicted frequencies of diabetes were compared among patients being treated for tuberculosis during the period 1967-74. Of 43 patients with tuberculosis, 65 percent had diabetes, whereas the age and sex adjusted expected frequency was 33 percent. This association is highly significant ($p<0.01$).

9. Development of a comprehensive care program for Indian diabetics: Diabetes is a major health problem of many American Indian tribes. Previous studies have demonstrated that improved care can reduce both acute and some chronic complications of diabetes. An attempt has been made to develop a comprehensive care program coordinating efforts of physicians, nurses and other medical personnel and including a patient education program tailored to the needs of this community. The education program has been designed for easy adaptation to various groups in the community. It may be modified for utilization among other Indian groups. It can also serve as a model for care and education programs dealing with other chronic diseases.

10. HL-A typing and diabetes: Recent publications have claimed associations of HL-A antigens and diabetes. We have initiated a study to obtain HL-A and LD typing on all Pima diabetics and normals over the age of 55 years to determine whether such associations exist in this population.

Significance of Research: The relationship between serum insulin levels and glucose tolerance have shown that much of the controversy concerning the characteristics of insulin secretion in mild carbohydrate intolerance has resulted from the wide variation in levels and the fact that the mean insulin levels vary considerably over a narrow range of glucose tolerance.

The question of whether abnormalities in insulin secretion precede the onset of unequivocal glucose intolerance has been examined and no differences in insulin response to an oral glucose load were found in true prediabetic subjects, suggesting that islet β cell dysfunction is not present prior to the onset of glucose intolerance.

Since obesity is a factor known to change peripheral resistance to insulin, a more detailed analysis of its development and significance is required. Preliminary results indicate that the development of obesity among Pima Indian children starts at about the age of 6 years, and from age 11 years onwards Pima Indians are approximately 30 percent heavier than Caucasians.

Analysis of sequential glucose tolerance testing provides important information about factors which are associated with the development of diabetes. Such analysis also allows better definition of those subjects who will remain diabetic and those who have indeterminate degrees of glucose tolerance.

Proposed Course: The long-term prospective studies of diabetes and its vascular complications will be continued. Since many subjects have now had documented glucose intolerance for ten years, detailed analyses of the factors related to the incidence, rather than prevalence, of the specific complications will be initiated. Such analyses offer a much greater likelihood for identification of factors of causal significance than is possible with prevalence data alone.

Further studies to characterize factors related to the onset of glucose intolerance will also be undertaken and will include determination of the relationship of insulin secretion and obesity to the evaluation of glucose intolerance.

Keyword Descriptors: diabetes mellitus, natural history, American Indian

Honors and Awards: None

Publications:

1. Savage, P.J., Bennett, P.H., Gorden, P., and Miller, M.: Insulin Responses to Oral Carbohydrate in True Prediabetics and Matched Controls. Lancet No. 7902, I, 300-302, 1975.

2. Savage, P.J., Dippe, S.E., Bennett, P.H., Gorden, P., Roth, J., Rushforth, N.B., and Miller, M.: Hyperinsulinemia and Hypoinsulinemia - Insulin Responses to Oral Carbohydrate Over a Wide Spectrum of Glucose Tolerance. Diabetes 24: 362-368, 1975.

3. Rushforth, N.B., Bennett, P.H., Steinberg, A.G., and Miller, M.: Comparison of the Value of the Two- and One-Hour Glucose Levels of the Oral GTT in the Diagnosis of Diabetes in Pima Indians. Diabetes (in press).

4. Hamman, R.F., Bennett, P.H., and Miller, M.: The Effect of Menopause on Serum Cholesterol in American (Pima) Indian Women. Am J Epidemiol (in press).

Project No. Z01 AM 69,001-06 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Prospective Study of the Complications and Outcome of Diabetic and Prediabetic Pregnancies in the Gila River Indian Community

Previous Serial Number: NIAMDD-EFS-5(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen L. Aronoff
Dr. Peter J. Savage
Dr. Richard F. Hamman

Cooperating Units: Indian Health Service

Man Years:

Total:	0.5
Professional:	0.2
Others:	0.3

Project Description:

Objectives: To determine whether pregnancies in prediabetic women are associated with excessive complications during pregnancy or with excessive mortality or morbidity in the offspring.

Methods Employed: The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) are followed and recorded in a standardized manner.

All patients in the prenatal clinic of the Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. These patients receive another modified glucose tolerance test 24-48 hours post partum and are retested at approximately two yearly intervals to document the development or lack of development of diabetes. Infants will be examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. At age five years the children will be reexamined to detect any further abnormalities which may have been inapparent at birth.

Major Findings: None. Further analysis has been deferred pending collection of additional data.

Significance of Research: In view of the high prevalence of diabetes in the Pima Indians, studies in the Gila River Community provide a unique opportunity to evaluate the outcome of both prediabetic pregnancies and pregnancies with mild carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it is well established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased prevalence of fetal mortality and maternal morbidity, it is not certain whether treatment will improve the outcome of those pregnancies in which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance have so far yielded conflicting results. With the recent adoption by the Indian Health Service of more stringent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that this study will be able to add to the evaluation of this problem by monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course: The pregnancy study will continue in conjunction with the study of the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Keyword Descriptors: diabetes mellitus, complications, pregnancy

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,003-02 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evaluation of the Prediabetic State in the Pima Indians

Previous Serial Number: NIAMDD-EFS-8(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen L. Aronoff
Dr. Phillip Gorden
Dr. Jesse Roth
Dr. Marvin Siperstein
Dr. Roger Unger
Dr. Joseph Williamson

Cooperating Units: Phoenix Indian Medical Center
Diabetes Branch, University of California Medical School at San Francisco
University of Texas-Southwestern Medical School at Dallas
Washington University School of Medicine

Man Years:

Total:	0.8
Professional:	0.5
Others:	0.3

Project Description:

Objectives: The natural history of diabetes mellitus has yet to be well elucidated. A prospective study of the genetically suspect prediabetic subject (defined as the non-diabetic offspring of two diabetic parents) appears to be the most direct and reliable method of describing the natural history of the disease.

Study of the prediabetic will enable us to better understand the interrelationship of hyperglycemia and microangiopathy which is presently highly controversial and poorly defined. This is possible through the study of chemical and physical abnormalities in prediabetics. Chemical abnormalities may include subtle changes in glucose and insulin as well as possible specific urinary protein abnormalities. The major physical abnormality under investigation is the muscle capillary basement membrane thickness.

Possible capillary basement membrane thickening, prior to glucose intolerance has been reported by Siperstein, but challenged by Williamson. Variation in findings in part are due to variation in techniques. Resolution of the conflict is of utmost importance in understanding the interrelationship of glucose tolerance and microangiopathy.

The purposes of the study are:

1. To study whether or not carbohydrate abnormalities, insulin abnormalities, capillary basement membrane hypertrophy and urinary protein abnormalities are characteristic of prediabetes.

2. To compare the two major techniques of basement membrane measurement.

3. To initiate a long-term prospective study of prediabetes.

Methods Employed: Two groups have been selected for study - the non-diabetic offspring of two diabetic parents and the non-diabetic offspring of two normal parents.

The two groups are matched for age, sex and percent desirable weight and following a dietary preparation were admitted to the Clinical Research Center for testing. Tests include an oral glucose tolerance test, intravenous glucose tolerance test, cortisone glucose tolerance test, an arginine infusion, a quadriceps needle muscle biopsy, fundus photographs and 24-hour urine collection for determination of specific urinary proteins by immunodiffusion and characterization of volatile organic compounds.

Results:

1. Arginine Infusion: Evaluation of glucose, insulin and glucagon response to arginine infusion revealed that normal (N=27) and prediabetic (N=35) Indians responded similarly. Glucagon and insulin response was not significantly different at any time, while the glucose response to arginine in the prediabetics was slightly but significantly greater at 30 and 40 minutes after initiation of the infusion. Fasting glucagon was 126±10 pg/ml in prediabetics vs. 119±11 pg/ml in normal Indians. At 40 minutes after initiation of infusion the levels were 295±14 pg/ml in prediabetics as compared to 253±19 pg/ml in normal Indians.

   Prediabetes in Pima Indians is not characterized by hyperglucagonemia to arginine infusion.

2. Intravenous Glucose Tolerance Test: Insulin and glucose responses were measured in 27 normal and 35 prediabetic Indians. Peak insulins occurred 5 minutes after starting infusion in both groups and were 180±12 μU/ml in prediabetics and 180±12 μU/ml in normal Indians.

Peak glucose levels were not significantly different in the two groups, but K-values were significantly lower in prediabetic (1.65±12) than in normal Indians (2.36±.23) ($p < .05$). Thus, prediabetic and normal Pima Indians have similar acute phase insulin release to IV glucose. Diminished or delayed insulin release is not characteristic of prediabetes in Pima Indians.

3. Cortisone Glucose Tolerance Test: The frequency of abnormal cortisone glucose tolerance tests (CGTT) was compared in the normal and prediabetic Indians. Nine of twenty-seven normal Indians had an abnormal CGTT while 20 of 35 prediabetic Indians had an abnormal test. This difference in frequency was significant ($p < .05$). The significance of this finding can only be answered by long-term follow-up.

4. Specific Urinary Protein Abnormalities: Not yet available.

5. Capillary Basement Membrane Thickness: Not yet available.

6. Characterization of Urinary Volatile Organic Compounds: Not yet available.

Proposed Course: Over 60 subjects have been studied and all samples collected. Analysis is underway on the results that have been completed.

Results of capillary basement membrane thickness, specific urinary protein abnormalities and characterization of urinary volatile organic compounds should be available by late 1975.

Keyword Descriptors: capillary basement membrane, insulin, prediabetes

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,004-02 EFSB
1. Epidemiology and Field Studies Br
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evaluation of the Prediabetic State in the Pima Indians and Caucasian Controls

Previous Serial Number: NIAMDD-EFS-10(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen L. Aronoff
Dr. Richard F. Hamman
Dr. Peter J. Savage
Dr. Jesse Roth
Dr. Phillip Gorden
Dr. Marvin Siperstein
Dr. Joseph Williamson
Dr. Roger Unger

Cooperating Units: Phoenix Indian Medical Center
University of California Medical School at San Francisco
University of Texas-Southwestern Medical School at Dallas
Washington University School of Medicine

Man Years:

Total:	0.7
Professional:	0.2
Others:	0.5

Project Description:

Objectives: Identification of abnormalities in prediabetic individuals has been a major goal of diabetes research in attempting to understand the natural history of the disease.

Tests proclaimed as diagnostic of the prediabetic state include the cortisone glucose tolerance test, time and quantity of insulin release in relation to glucose loads and capillary basement membrane thickness.

These tests were conducted on a group of genetically normal and genetically prediabetic Pima Indians. The very high prevalence of diabetes in the Pima people, while providing a very large group of genetically prediabetic individuals, raised a problem of finding individuals who are truly normal. Genetically normal Pima offspring were selected from genetically normal parents 45 years of age or older. It is conceivably possible, however, that with the high prevalence of diabetes in the tribe, virtually all the Pimas could carry the diabetic genome.

A group of non-diabetic Caucasians with non-diabetic family histories were examined as an additional control group to compare with the "normal" Pimas.

Methods Employed: The study population was selected from non-diabetic Caucasians with non-diabetic family histories who are matched in age, sex and weight with normal Pima volunteers. Each person was admitted to the Clinical Research Center for testing. Tests included an Oral Glucose Tolerance Test, Arginine Infusion, Cortisone Glucose Tolerance Test, Intravenous Glucose Tolerance Test, a quadriceps needle muscle biopsy and a 24-hour urine collection which is being analyzed for specific urinary proteins by immunodiffusion and characterized for volatile organic compounds.

Results:

1. Arginine Infusion: Fasting glucagon in normal Indians (119±11 pg ml) was not found to be significantly different from Caucasians (148±14 pg/ml). Small differences between fasting glucose occurred between the two groups but the mean maximal rise was similar. Fasting insulin and arginine stimulated insulin was significantly greater in the normal Indians.

   Glucagon levels in the normal Indians and Caucasians were similar at all points reaching a 40 minute peak of 253±19 pg/ml in the normal Indians and 270±24 pg/ml in the Caucasians.

   Thus, normal Pima Indians and normal Caucasians have similar arginine stimulated glucagon response.

2. Intravenous Glucose Tolerance Test: In response to a rapid infusion of intravenous glucose normal Indians reached a peak glucose of 266±12 mg/dl at 5 minutes vs. 256±10 mg/dl at 5 minutes for Caucasians. K values in the two groups were also similar being 2.36±.23 for normal Indians and 1.99±.12 for normal Caucasians. Fasting insulins in the normal Indians (31±3 μU/ml) were significantly higher ($p<.0001$) than in Caucasians (16±1 μU/ml). Peak insulins were reached at 5 minutes in both groups and were 180±12 μU/ml in Indians and 75±6 μU/ml in Caucasians ($p<.0001$).

   Only part of the difference in insulin response could be explained by the heavier weight of the Indians (142±6% desirable weight) as compared to the Cuacasians (116±4% desirable weight). Since both groups were instructed in similar diets for the 3-4 days prior to the test, this is also not a totally satisfactory explanation. Presently, most of the difference in insulin response must be attributed to unknown factors including possibly race.

3. Capillary Basement Membrane Thickness: Not yet available.

4. Specific Urinary Proteins: Not yet available.

5. Volatile Urinary Organic Compounds: Not yet available.

Significance: Characterization of the prediabetic state may allow an understanding of the basis of diabetes mellitus. Because of the ability to control many variables it is hoped that this study will allow the demonstration of the earliest lesions of diabetes mellitus.

Proposed Course: Data has been collected on 35 prediabetic subjects. Individual's photomicrographs for basement membrane thickness and urine for specific urinary proteins and volatile organic compounds are now being analyzed. Results should be available by late 1975.

Keyword Descriptors: glucagon, insulin, prediabetes, capillary basement mem.

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,005-02 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Evaluation of Basement Membrane Thickness in Diabetic Pima Indians

Previous Serial Number: NIAMDD-EFS-9(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen L. Aronoff
Dr. Richard F. Hamman
Dr. Peter J. Savage
Dr. Roger Unger
Dr. Marvin Siperstein
Dr. Joseph Williamson

Cooperating Units: Phoenix Indian Medical Center
Sacaton Indian Health Service Hospital
University of California Medical School at San Francisco
Washington University School of Medicine

Man Years:

Total:	0.8
Professional:	0.3
Others:	0.5

Project Description:

Objectives: Since Siperstein first reported finding thickened muscle capillary basement membranes in diabetics, multiple studies have been performed in an attempt to evaluate his results. Findings have varied remarkably from one investigator to another to the extent that while muscle capillary basement membrane thickening (MCBMT) is generally accepted as characteristic of diabetes, the frequency of its occurrence, its relationship to age, sex, duration of diabetes, and complications of diabetes is now highly controversial.

The contradictions in results appear to be related, at least in part to varying techniques of fixation and measurement employed by different investigators.

A study of MCBMT in normal and prediabetic Pima Indians is presently underway. MCBMT in diabetic Pima Indians has never been quantitated and it is not known if thickening occurs to the same extent in them as in non-Indians.

The major purposes of the study are:

1. To determine whether Pimas with diabetes develop capillary basement membrane thickening.

2. To determine whether such thickening is related to the duration of carbohydrate intolerance and to the complications of diabetes.

3. To compare capillary basement membranes of diabetic Pimas with the MCBM of normal and prediabetic Pima Indians and normal non-Indians.

Methods Employed: Two groups of diabetics were examined a) long-term diabetics, defined as subjects with fasting plasma glucose ≥ 140 mg/100ml or a two hour post-carbohydrate load glucose ≥ 300 mg/100ml of >10 years duration with and without retinopathy and b) short-term diabetics, defined as subjects who have had a documented marked deterioration in their oral glucose tolerance over the last 5 years from a two hour post-carbohydrate load plasma glucose <140 mg/100ml to a fasting plasma glucose ≥ 140 mg/100ml or a two hour post-carbohydrate load glucose ≥ 300 mg/100ml.

Subjects had an oral glucose tolerance test, a quadriceps needle muscle biopsy, a 24-hour urine collection for specific urinary protein determination and identification of volatile organic compounds as well as fundus photographs and direct fundus examination. Those subjects designated as recent-onset diabetics also received an arginine infusion.

Results:

1. Arginine Infusion: Glucose, insulin and glucagon were measured before and during arginine infusion in Pima diabetics. Fasting glucagon level (mean ±SEM) was 148±14 pg/ml in diabetics as compared to 119±11 pg/ml in normal Indians. This was not significantly different. During arginine infusion glucagon levels rose more rapidly to a peak of 399±28 pg/ml in diabetics as compared to 253±19 pg/ml in normal Indians ($p<.001$). Fasting and stimulated insulin response to arginine infusion was not significantly different at any time between the normal and diabetic Indians.

   If diabetics were subdivided into those with more severe diabetes who had previously received insulin (N=6), but did not have interfering insulin antibodies (N=5), and those subjects with less severe diabetes (N=6), in the more severe diabetics fasting glucagon (158±24 pg/ml) and glucose (227±21 mg/dl) were higher but not significantly different from the glucagon (132±13 pg/ml) and glucose (172±26 mg/dl) in the milder diabetics.

The glucagon and glucose response to arginine infusion in the two groups was not significantly different at any point and the milder diabetic reached a peak of 409±23 pg/ml vs. 388±54 pg/ml in the more severe diabetics. However, fasting insulin in the milder diabetics (37±6 μU/ml) was significantly higher than in the more severe diabetics (16±3 μU/ml) and during infusion the insulin levels in the milder diabetic reached a significantly higher peak (111±16 μU/ml) than the more severe diabetics (53±12 μU/ml) ($p<.05$) and were significantly higher at all times.

Diabetic Pima Indians demonstrated hyperglucagonemia in response to arginine infusion as has previously demonstrated in non-Indian groups. Also, the finding that fasting and arginine stimulated glucagon levels were not different in milder diabetics with high insulin levels and the more severe diabetics with low insulin levels strongly infers that the hyperglucagonemia of diabetes is unrelated to the level of plasma insulin.

2. Capillary Basement Membrane Thickness: Not yet available.

Significance of Research: This study is expected to resolve the conflict concerning the optimal method of capillary basement membrane measurement. It also will better define the relationship between the length of diabetes, diabetes complications and MCBM thickening.

Proposed Course: All subjects have been studied. Preparation of photomicrographs and measurement of each individual's basement membrane thickness is presently underway and all results should be available by late 1975. Analysis of specific urinary proteins and volatile organic compounds in the urine are also underway.

Keyword Descriptors: diabetes mellitus, muscle capillary basement membrane

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,006-05 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Gila River Indian Community Autopsy Study

Previous Serial Number: NIAMDD-EFS-6(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Joseph Likos
Dr. Richard F. Hamman
Dr. Stephen L. Aronoff
Dr. Peter J. Savage
Dr. Philip LeCompte
Dr. Max Miller
Dr. Delbert Eatough

Cooperating Units: Indian Health Service, (PHS)
Veterans Administration
Case Western Reserve University
Brigham Young University

Man Years:

Total:	1.0
Professional:	0.3
Others:	0.7

Project Description:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Research in Progress:

1. Renal Pathology: Review of the histologic data from 105 Pima Indian autopsies indicated that the pathology found in the kidneys of diabetic Pima was the same as in diabetics of other races.

   Diabetic renal glomeruli may be larger than those from non-diabetic kidneys, but little systematic study of glomerular size or variability has occurred. A comparison of the renal glomerular size of Pima diabetic and non-diabetic kidneys with those of other groups has been initiated.

   Preliminary results from Pima subjects indicate that renal glomeruli were slightly larger than those of white patients, and that diabetic glomeruli were larger than those of non-diabetic Pima controls. An analysis of the hydroxyproline content of these glomeruli is underway.

2. Trace Metals: The high prevalence of diabetes, makes the Pima Indians an ideal group for the evaluation of trace metal disease relationships. The analysis of post mortem frozen tissues has been performed. Using atomic absorption and x-ray fluorescent spectroscopy, tissue levels of Cr, Mn, Ca, Mg, Li, Fe, Cu, Zn, and other metals will be described. The liver, pancreas, kidney, fat, hair, aorta and spleen will be analyzed from subsequent autopsies. Initial results suggest an age related rise in pancreatic chromium levels, but similar levels in diabetics and non-diabetics.

3. Histopathology of the pancreas: Because of the interrelationship of pancreatic β cells, insulin secretion and diabetes, the quantification and characterization of α and β cells of the pancreas using scanning microscope and special staining methods has been initiated.

Significance of Research: As more subjects who have been under long-term ante-mortem surveillance are autopsied, more precise clinical pathologic correlations will be available for the study of diabetes mellitus than has previously been possible.

The verification of the interracial similarity of diabetic nephropathy allied with work now in progress to determine renal glomerular tuft size may help confirm that the disease process in the Pima can serve as a model from which to project findings to all diabetics.

Quantification of pancreatic β cells may be used to further define etiologic hypotheses for the role of insulin production and secretion in diabetes mellitus.

The Pima are the first population with diabetes under continued surveillance in which trace metals concentrations have been investigated. Correlation with ante-mortem clinical data and autopsy findings may help elucidate risk factors associated with the development of diabetes, atherosclerosis and other disease entities.

Proposed Course: The systematic collection and cataloging of tissues will continue. As sufficient specimens become available, detailed examinations of various tissues will be made. Further studies of the kidney, including electron microscopy and biochemical quantification of the α and β cells of the islets of Langerhans will be the major activity in the coming year.

Keyword Descriptors: autopsy, diabetes mellitus, histology, trace metals

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,007-08 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Prevalence of Diabetes in Indian Populations in the Southwestern United States

Previous Serial Number: NIAMDD-EFS-1(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: None

Cooperating Units: Indian Health Service, (PHS)

Man Years:

Total:	0.6
Professional:	0.3
Others:	0.3

Project Description:

1. To determine the prevalence of diabetes among various native populations in the Southwestern United States.

2. To formulate and test hypotheses which may explain the high frequency of diabetes mellitus among some of the populations.

Methods Employed: Random samples of total populations will be examined by means of standardized tests of glucose tolerance to enable the prevalence of glucose intolerance to be determined. Other examinations will be performed to document the frequency of disorders when appropriate.

Major Findings: Completion of the San Carlos Apache Survey in late FY 1974 allowed comparison of most major tribal groups in the Southwest, using identical survey methods. Major differences in the prevalence of diabetes among related and unrelated tribal groups have been found.

Significance of Research: These findings suggest that the prevalence of diabetes in populations may be environmentally determined as well as being related to the genetic origins of the populations.

Proposed Course: The survey data and genetic markers (blood groups, albumin phenotyping and others) will be analyzed to evaluate genetic and environmental correlation of diabetes among American Indian populations in this region.

Keyword Descriptors: diabetes mellitus, epidemiology, American Indian

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,008-03 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Coxsackie B4 Virus Infection and its Relationship to Diabetes Mellitus

Previous Serial Number: NIAMDD-EFS-7(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. James Maynard
Dr. Ken Berquist

Cooperating Units: Indian Health Service
Center for Disease Control

Man Years:

Total:	1.0
Professional:	0.3
Others:	0.7

Project Description:

Objectives:

1. To determine the prevalence and incidence of diabetes mellitus five years after an epidemic caused by Coxsackie B4 virus.

2. To evaluate the relationships of serum lipids and blood pressures to diabetes and cardiovascular abnormalities.

Major Findings: In recent years Coxsackie B4 infection has been implicated as a possible etiologic agent in the development of diabetes mellitus. There have been both retrospective epidemiologic evaluations and animal studies which support the concept that Coxsackie B4 virus can cause hyperglycemia. In 1967 an epidemic occurred on two islands in the Bering Sea which was identified serologically and by virus isolation to be Coxsackie B4.

An attempt was made to evaluate whether diabetes developing in the subsequent five years was related to Coxsackie B4 infection.

Coxsackie B4 Infection: Following the 1967 epidemic 77 percent of the subjects had a positive titer to Coxsackie B4 and in the younger age groups it approached 100 percent. Five years later, 61 percent of the males and 63 percent of the females had detectable levels of antibody present.

No significant association between the two hour plasma glucose level and Coxsackie B4 neutralizing antibody titers could be demonstrated.

Significance of Research: Since these islands experienced an epidemic of Coxsackie B4 in a previously virgin population, they presented an ideal test to establish whether or not Coxsackie B4 is indeed a causal agent for the development of diabetes.

Five years following the epidemic no relationship between new causes of diabetes and earlier Coxsackie B4 infection could be demonstrated with the possible exception of the 45-54 age group. The significant difference found in this age group probably represents a chance finding. Previous evidence suggested that the strongest association would likely occur among juvenile onset of diabetes. No case of diabetes occurred in any person less than 25 years of age following the epidemic among the subjects in this study.

Coxsackie B4 over a five year period did not cause the development of diabetes in this population in those aged under 25 years. Although diabetes did occur following the epidemic in the adult population, no evidence that Coxsackie B4 infection was the cause was found. The findings have been prepared for publication.

Proposed Course: None

Keyword Descriptors: Coxsackie B4, diabetes mellitus

Honors and Awards: None

Publications:

Dippe, S.E., Bennett, P.H., Miller, M., Maynard, J.E., and Berquist, K.R.: Coxsackie B4 Virus Infection and Diabetes - Failure to Demonstrate a Causal Association in Man. Lancet (in press).

Project No. Z01 AM 69,009-10 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1975 through June 30, 1975

Project Title: Prospective Study of the Natural History of Arthritis and Rheumatism in the Gila River Indian Community

Previous Serial Number: NIAMDD-EFS-3(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Thomas A. Burch
Dr. Peter J. Savage
Dr. Richard F. Hamman
Dr. Stephen L. Aronoff
Dr. Jesse Jupiter

Cooperating Units: Indian Health Service
Hawaii State Health Department

Man Years:
Total: 1.0
Professional: 0.5
Others: 0.5

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predicative and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods Employed:

1. The following tests and examinations will be repeated on all Indians five years of age or over, living on the Sacaton Service Unit portion of the Gila River Reservation.

   a. Clinical interview
   b. Physical examination
   c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
   d. Laboratory determinations: rheumatoid factor, uric acid

2. The data from the above examinations and tests as well as demographic, pedigree, environmental information, and data on other conditions and diseases will also be collected.

Major Findings:

All Pima Indians with previously identified evidence of bilateral sacro-iliitis have been reviewed and HL-A typing has been obtained in them. An equal size group of sex matched controls have also been examined by HL-A typing. The present data indicate that approximately 50 percent of the persons with bilateral sacro-iliitis have HL-A W27 antigen compared to a population prevalence of approximately 10 percent. The difference is highly statistically significant. The data are being analyzed to determine whether or not there are any particular characteristics of those persons who demonstrate the association between HL-A W27 and sacro-iliitis; for example, whether or not those who are W27 positive manifest classical, clinical ankylosing spondylitis more frequently than the others.

Significance of Research:

Since a strong association between HL-A W27 and ankylosing spondylitis has been demonstrated in Caucasians, it is important to determine whether the geography of ankylosing spondylitis, which has been previously described among the Pima Indians and eight other populations is due primarily to variation in the frequency of HL-A W27 antigen among these populations. The fact that only one-half of the subjects with bilateral sacro-iliitis have the antigen suggest that either there are other diseases which are associated with bilateral sacro-iliitis other than ankylosing spondylitis or that the locus for the genetic determination of bilateral sacro-iliitis is somewhat removed from the HL-A W27 locus.

Proposed Course:

As the HL-A typing on the Pima Indian population is being extended it should be possible to determine the risk of developing sacro-iliitis among those who are W27 positive on an age, sex specific basis. These data will be valuable in determining the risk of developing ankylosing spondylitis and in the identification of possible participating factors in those presumed to be susceptible to the disease on a genetic basis. It is hoped that this may permit the identification of specific environmental factors responsible for appearance of this disease.

Keyword Descriptors: arthritis, rheumatism, natural history, American Indian

Honors and Awards: None

Publications:

Henrard, J-C., Bennett, P.H., and Burch, T.A.: Rheumatoid Arthritis in the Pima Indians of Arizona: An Assessment of the Clinical Components of the New York Criteria. Int J Epidemiol (in press).

Project No. Z01 AM 69,010-09 EFSB
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Southwestern Indian Gallbladder Study

Previous Serial Number: NIAMDD-EFS-4(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: None

Cooperating Units: Indian Health Service

Man Years:

Total:	0.1
Professional:	0.1
Others:	0

Project Description:

Objectives:

To determine whether cholesterol gallstones may be dissolved effectively and safely by the oral administration of bile salts.

Methods Employed:

1. Adult Indian subjects who have asymptomatic gallstones will be identified.
2. Such subjects will be asked to participate in a double blind controlled study of the effect of oral administration of bile salts in producing the dissolution of the gallstones over a two-year period.

Major Findings: None

All activity this year has been directed towards attempting to define a mechanism to carry out the protocol which has been developed for the National Cooperative Gallstone Study.

Significance of Research:

Chenodeoxycholic acid has been demonstrated to be an effective means of dissolving radiolucent gallstones in persons with a functioning gallbladder. The safety and side effects of this mode of therapy have not yet been adequately documented in a large patient series, nor in American Indians. The multicenter cooperative trial will result in such information and result in the development of the specific indications for bile salt therapy versus cholecystectomy in the treatment of cholelithiasis.

Proposed Course:

Since the number of available personnel to conduct the study will be reduced in the forthcoming year, the conduct of the trial among the Pima Indians has been abandoned. The project is discontinued.

Keyword Descriptors: gallbladder, American Indian

Honors and Awards: None

Publications: None

Project No. Z01 AM 69,011-07 EFSB
Epidemiology & Field Studies Branch
Metabolic Diseases Epidemiology Unit
Bethesda

PHS-NIH
Individual Project Report
July 1, 1974 through June 30, 1975

Project Title: Experimental and Field Studies of Iodine Metabolism and Endemic Goiter

Previous Serial Number: NIAMDD-EFS-11(c)

Principal Investigator: Robert L. Vought, M.D., M.P.H.

Other Investigators: Carol A. Mapes, Ph.D.
Freddie A. Brown, B.S.

Cooperating Units: Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMDD

Man Years:

Total:	3 1/4
Professional:	2 1/4
Other:	1

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defects responsible for the disease. The immediate short-term objectives are to identify and characterize certain microbially-produced goitrogens.

Methods Employed: Accomplishment of the objectives has involved bench laboratory research, clinical research and collaborative field studies. The current year has been devoted to development of an assay for goitrogens *in vitro* and to further identify biochemical and immunological properties of the semi-purified goitrogen derived from *Escherichia coli*. The *in vitro* assay has now been satisfactorily developed and is being utilized for the purposes just described.

Major Findings: The antithyroid compound derived from *E. coli* has now been definitely characterized as a true goitrogen by showing that it blocks the organification of radioiodine in rat thyroid. Biologic and immunologic studies have also shown that the partially-purified material is immunogenic as well as goitrogenic. Rabbits injected with the goitrogen produced antibodies which block the activity of the goitrogen *in vivo* and *in vitro*. The active portion of the material is apparently particulate matter and its further chemical purification and characterization will require additional biochemical studies. Sheep blood cells coated with this antigenic material can form the basis of a hemagglutination test which should be capable of measuring antibody to the goitrogen in human and animal sera.

Keywords: Iodine, iodine metabolism, goiter, endemic goiter, goitrogens, goitrogen assay for *E. coli*, and thyroid.

OFFICE OF SCIENTIFIC AND TECHNICAL REPORTS

Summary

Fiscal Year 1975

The Office of Scientific and Technical Reports, formerly the Office of Public Information, prepares and disseminates most of the regular and special program reports required of the NIAMDD by the Congress, and by the NIH and the DHEW. Its staff members also assist in preparation of staff papers, professional publications, and Congressional reports, and responds to inquiries of all types.

Both the scope and depth of the Institute's categorical research programs have increased rapidly in recent years, and reporting on all of these varied and complex activities has made heavy demands on the relatively small office staff. The OSTR services the general, professional, Governmental and Congressional publics by means of the mass media, specialized media, and by individual communications.

Scientific and Technical Reports:

A major effort of the OSTR each year is the preparation of comprehensive special reports and related material in connection with Congressional appropriation hearings. In FY 1975 special reports were prepared on arthritis, diabetes, digestive diseases, kidney disease, hematology, cystic fibrosis and nutrition. Such reports invariably have tight deadlines and call for a concerted effort by OSTR staff members.

Several special report subjects, such as alcoholism, concern the research conducted by a number of Institutes and require collaboration with staff members of other involved Institutes. Other reports include the NIAMDD Director's opening statement and the so-called budget narrative, the latter being a very detailed and voluminous undertaking. Still another report, "Highlights of Research," was absorbed in FY 1975 by a new publication entitled "Research ADVANCES - 1975." It is anticipated that this booklet, which reflects research accomplishments by the entire NIH community, will be an annual publication that will replace Highlights of Research.

The so-called "weekly report," which briefly summarizes significant intramural and extramural research findings, is utilized primarily as a basis for many of the regular and special program reports required of the NIAMDD. It is also distributed in response to requests from the various publics serviced by the OSTR.

Characteristic weekly reports during the past fiscal year included determination of the chemical structure of follicle stimulating hormone; successful treatment of psoriasis with orally administered methoxsalen and exposure to a new light system; significant reversal of the diabetic state in monkeys following implantation of intact pancreatic islets; an acute decrease in blood glucose levels in diabetic patients resulting from administration of somatostatin, and demonstration of an increased prevalence of the histocompatibility antigen W27 in patients with juvenile rheumatoid arthritis, suggesting an etiologic link with Reiter's disease and ankylosing spondylitis.

Weekly report production has been adversely affected in recent years by the ever-increasing number of Congressional letter inquiries which take priority over preparation of the weekly report. For example, 98 such inquiries were handled by the OSTR in calendar year 1969, while 189, an increase of more than 90 percent, were handled in calendar year 1974.

Freedom of Information Responsibilities

The 1974 Amendments to the Freedom of Information Act have meant new responsibilities for the staff of the Office of Scientific and Technical Reports, which is responsible for implementation of this legislation within the Institute. A staff member serving as Freedom of Information Officer executes, maintains and administers the FOI Index, as required by the new law, and prepares quarterly reports on the availability of required Index materials. The FOI Officer evaluates all requests by Institute personnel to deny public access to information, advises and counsels Institute staff on all aspects of FOI, and serves as Institute liaison between program officials, general counsel staff and the NIH Freedom of Information Officer.

Although the 1974 Amendments have only been in effect since February 19, 1975, the OSTR has established the FOI Index and responded to many requests from Institute staff members for advice on various aspects of FOI policy or assistance in determining whether requested materials meet the requirements of confidentiality necessary to withhold them under the Act. The OSTR has also taken initiatives to inform Institute staff members of the impact and implications these revisions in the law will have on the work of the Institute.

Press Activities:

In FY 1974 press and related activities were largely centralized within the office of the Associate Director for Communications, NIH. The OSTR, nevertheless, prepared and issued a representative number of press releases, in line with established policy to publicize the NIAMDD's research activities and programs.

Representative press releases included the following: (1) the awarding of $4 million in grants to establish four Diabetes-Endocrinology Centers; (2) the conclusion, based on studies of the Skylab astronauts, that manned space flights of several years duration would have serious effects on bone and muscle unless protective measures can be developed; (3) appointment of ten public members, including six medical scientists and four laymen, of the newly created seventeen-member National Commission on Diabetes, and (4) a warning from the NIAMDD and the American Diabetes Association concerning a possible optimistic "misunderstanding" on the part of the American public regarding the status of transplantation of the pancreas and pancreatic tissues as a "cure" for diabetes.

Radio and Television:

In conjunction with the NIH Office of Information, a number of radio and television news spots and announcements were prepared and released concerning the availability of NIAMDD publications. The public response to these notices has necessitated the services of a "Stay-in-School" employee to work parttime to handle the backlog of requests.

The OSTR also arranged for the appearance of several NIAMDD scientists on radio and television programs, including Panorama, the Fred Fiske Show, Everywoman, etc.

Public Inquiries:

Inquiries from the general, professional and Governmental publics take many forms. Each, however, receives a prompt individualized reply, which often is supplemented by written informational material. If referral to another agency for additional information is indicated, this is done swiftly and courteously. Considerable staff time is allotted to answering both telephone and letter inquiries from ill individuals, their friends and relatives, school children, physicians and scientists.

In FY 1975 the OSTR staff responded to more than 2400 such inquiries, excluding the many thousands of routine requests for publications.

Congressional Inquiries:

Telephone and letter inquiries from the Congress, which have increased steadily in recent years, receive special attention from the OSTR staff. Each telephone inquiry, for example, is documented by a memorandum detailing the nature of both the request and the response. Letter inquiries, on the other hand, are "logged in" and carefully worded replies are forwarded to the Congressman within the allotted time span.

Characteristically, Congressional inquiries concern ill constituents in need of medical or financial assistance, funding of specific NIAMDD research programs, or the status of a particular research grant application. During FY 1975 OSTR staff members responded to 302 Congressional inquiries.

Publications:

Fiscal Year 1975 saw the issuance of several more public information "fact sheets" of diseases within the Institute's purview, including Scleroderma, Systemic Lupus Erythematosus, among others. A number of fact sheets will be revised in the next fiscal year to be incorporated into a new "Coping With..." series of pamphlets and brochures. In addition, booklets have been prepared on diabetes and arthritis, in conjunction with the new legislation concerning these disorders, for issuance in Fiscal Year 1975. The Institute's most popular publication, Facts About Nutrition, is being sent to more than 60,000 junior and senior high school libraries across the country, in collaboration with a project undertaken with the National Education Association. In FY 1975, more than 100,000 publications were distributed by the OSTR.

Exhibits:

The Office of Scientific and Technical Reports also conducts the NIAMDD program of scientific exhibits used primarily in professional education. Under severe travel and exhibit restrictions, the Institute was able to exhibit at only five medical and scientific meetings throughout the country, which were viewed by approximately 30,000 physicians, scientists and paramedical people. The Institute's exhibit on Cystic Fibrosis (revised) received the American Medical Association's Certificate of Merit at the June 1974 meeting in Chicago. Institute exhibits were also shown on the artificial kidney, human growth hormone, and general research both intramural and extramural. Various publications and reprints are usually distributed with these exhibits.

During the NIH Open House, the first in twenty years, the Institute's three major exhibits drew a large number of the 30,000 people who visited during the two day event. Of particular interest was an actual artificial kidney machine that was loaned to the Institute by Travenol. Information office staff members manned the exhibits and distributed thousands of pamphlets and brochures.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1975

Dr. G. Donald Whedon, Director
Mr. F. L. Mills, Executive Officer

The office of the Director has overall responsibility for directing and coordinating basic laboratory research; clinical investigations; extramural activities such as research grants, research fellowships, and training grants; contract research and developmental programs; and epidemiologic and clinical field studies. In addition, this office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

During FY-75 the Congress passed two pieces of legislation which directly affect the Institute. The National Diabetes Mellitus Research and Education Act, P.L. 93-354 was signed by the President on July 23, 1974. The act established the National Commission on Diabetes to formulate a long-range plan to combat diabetes mellitus and a Diabetes Mellitus Coordinating Committee to coordinate the research activites of the National Institutes of Health and other Federal health agencies having programs related to diabetes mellitus. The National Commission began holding hearings nation-wide with a March meeting at NIH. It is required to report to the Congress by December, 1975, a long-range plan to combat diabetes which will include the role NIAMDD should play in diabetes research. At the same time the Coordinating Committee has held two meetings and has begun to collect information on diabetes support from the member agencies.

The Congress also passed the National Arthritis Act of 1974, P.L. 93-640 in late December and the Act was signed by the President on January 4, 1975. This act establishes the National Commission on Arthritis and Related Musculoskeletal Diseases and an Arthritis Coordinating Committee. The Commission held its opening session April 7 and has seven months within which it must prepare a report to Congress on a long-range plan to combat arthritis. This plan will define the Institute's role in Arthritis research.

The Institute is lending its full support to the work of the Commissions and Coordinating Committees. Institute staff are actively working with these groups to help them achieve a timely resolution of their tasks. This has meant a commitment of both Institute labor and dollars in support. In addition, the Institute is actively recruiting an Associate Director for Arthritis and Related Musculoskeletal Disease as required by the Arthritis Act and an Associate Director for Diabetes, Endocrine and Metabolic Diseases as provided by the Diabetes Act while the Director serves actively on both the Commissions and the Coordinating Committees.

The Institute is also responsible for coordinating the US-USSR Cooperative Program in Arthritis. This involved two exchanges during the year, one by U.S. scientists to the Soviet Union early in the year and a return trip by the Soviets to the United States in May. Their visit included a tour of several major institutions across the U.S. plus a week long visit to NIH where they participated in a symposium on the role of viruses in autoimmune diseases and worked out a cooperative agreement for research in arthritis and related diseases. The US-USSR program brought a group of leading Soviet orthopedic surgeons to the U.S. in June. These scientists also toured the U.S. and participated at a major symposium on orthopedics held at the Homestead, Hot Springs, Virginia.

During the year only one section was abolished, the Section on Physiology and Clinical Nutrition in the Digestive Diseases Branch. Dr. Robert Gordon remains chief of the Branch. No branches, laboratories or sections were created during the year.

Important appointments to key positions within the Institute this year included in extramural programs, Dr. George T. Brooks, Associate Director for Extramural Program Activities; Dr. Harold Roth, Associate Director for Digestive Diseases and Nutrition; and Dr. Nancy Cummings, Associate Director for Kidney, Urologic, and Blood Diseases. In the area of Intramural Research Dr. Jun-ichi Tomizawa was appointed Acting Chief of the Laboratory of Molecular Biology and Dr. Cornelis J. P. Glaudemans began his appointment as Chief, Section on Carbohydrates, Laboratory of Chemistry. The Institute is continuing its search for qualified candidates for the position of Deputy Director, NIAMDD and for the positions of Associate Director for Arthritis, Bone and Skin Diseases and Associate Director for Diabetes, Endocrine, and Metabolic Diseases.

Volume of contract activity continued at a relatively level rate with a total obligation budgeted for the year at $7.4 million. Staff lost during FY-1973 was replaced by the addition of one negotiator and one clerk-typist. Artificial Kidney, Hormone Distribution, Scientific Communications, and augmentation of intramural research remained about the same. The clinical study of treatment of Crohn's Disease continued as did the clinical study of dissolution of gallstones by bile acid feeding.

RFP's were issued and contracts were awarded for endoscopy studies and for a clinical study for topical treatment of psoriasis by chemotherapeutic agents. A contract was awarded to study the current state of the art and determine the research needs in the interrelated fields of nephrology and urology. RFP's also were issued and two contracts were awarded: one for logistics support of the National Commission on Diabetes and one for similar support of the National Commission on Arthritis.

The activities of the Office of Scientific and Technical Reports are included in a separate report which precedes this summary. Efforts continue to provide an integrated program of scientific technical information and reports related to the Institute's program. Responses to 2,400 public inquiries were made during the year in addition to 302 Congressional inquiries which were made of this office. Over 100,000 publications of general interest were distributed.

The Basic Ideas in Biology Course has been temporarily discontinued because of the lack of a suitable instructor. Basic Ideas in Chemistry continues to be popular and successful, with two sections of Part IV completed in February. The classes stressed a practical understanding of the basic concepts underlying chemical research. A discussion of paper, thin-layer, and column chromatography was included. One section of a new course, Basic Mathematics for the Laboratory, was offered. Subjects included a review of math, the pH meter, weights and measurements, and the metric system. The classes are designed for employees who have little or no formal training in the sciences and emphasize a practical rather than theoretical approach. Upon completion of the training, supervisors are encouraged to utilize their employees' newly acquired skills. This Institute hopes to offer a continuation of the basic chemistry class and continue to offer the introductory chemistry class in the fall of 1975. During the year, short-term training opportunities were arranged for 85 secretarial-clerical employees, 21 supervisory managerial personnel and 163 members of the professional staff. This represents a substantial increase in the Institute's continuing commitment to the training of its staff to the fullest possible extent.

Equal Employment Opportunity

J. Harrison Ager, NIAMDD EEO Coordinator and Counselor, continued his recruiting activites by visiting minority colleges in Virginia and by attending the April FASEB meetings in Atlantic City, N.J. where he contacted the Organization of Black Scientists. The Institute also participated in the Minority and Women's Opportunity Resource Conference which had representatives from seventy-five ethnic, minority and women's academic institutions.

Mrs. Linnie Sloan started her three-year term as Institute representative on the NIH EEO council in September, 1974. Both Mr. Ager and Mrs. Sloan participated in training sessions at Hunt Valley and the NIH reservation which centered on the counselor's role in Affirmative Action and the use of the Federal Manual.

The Annual Institute EEO meeting was held April 9th. The first annual Institute EEO awards were presented to Dr. Whedon and Mr. Freddie Brown for their active roles in EEO. In addition each laboratory and branch held an EEO meeting during the year. These meetings focused primarily on the effects of management policy on NIH employees. The NIAMDD EEO Advisory Committee, chaired by the Director, continues to meet regularly to discuss all matters relating to the Affirmative Action Plan. The Affirmative Action Plan is currently being updated by the Committee. The Institute began a more active role in promoting EEO for women by meeting with the Federal Women's Program Coordinator to discuss the role of women in the NIH work force.

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